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CONTENTS OF VOLUME XIII

NUMBER 1

A Program of Analysis and Synthesis . . . . *W. C. Allee* 1

Lithium and Echinoderm Exogastrulation: With a Review of the Physiological-Gradient Concept . . . . *C. M. Child* 4

Regenerative and Accessory Growths in Planarians. II. Initiation of the Development of Regenerative and Accessory Growths . . . . *E. D. Goldsmith* 43

Somatic Effects of Temperature on Development in *Drosophila melanogaster*. I. Phenocopies and Reversal of Dominance . . . *G. P. Child, R. Blanc, and H. H. Plough* 56

Somatic Effects of Temperature on Development in *Drosophila melanogaster*. II. Temperature-Effective Period of Truncate . . . . . . *R. Blanc and G. P. Child* 65

The Heat Production and Oxygen Consumption of Pupae of *Galleria mellonella* at Different Constant Temperatures . . . . *Joy Bell* 73

Temperature Characteristics of the Electrical Potential in Frog Skin *T. Cunliffe Barnes* 82

The Action of Calcium on Muscle Protoplasm . . . . *L. V. Heilbrunn* 88

Mitotic Elongation. II. Osmotic and Salt Effects . . . . *Leon Churney* 95

Environmental Factors Affecting Cystment in *Woodruffia metabolica* *W. H. Johnson and F. R. Evans* 102

NUMBER 2

Unsolved Problems of General Biology . . . . *Ernest Everett Just* 123

Physiology of Development of the Feather. III. Growth of the Mesodermal Constituents and Blood Circulation in the Pulp . . . . *Frank R. Lillie* 143

The Control of Feather Color Pattern by Melanophores Grafted from One Embryo to Another of a Different Breed of Fowl . . . . *B. H. Willier and Mary E. Rawles* 177

The Localization of Pigment-forming Areas in the Chick Blastoderm at the Primitive-Streak Stage . . . . *Herbert L. Eastlick* 202

The Effect of Excess Potassium on the Cortex of the Fertilized Egg of *Arbacia punctulata* *Leon Churney and Floyd Moser* 212

The Effect of Thermal Increments on and the Subsequent Adjustment of the Protoplasmic Viscosity of *Amoeba proteus* . . . . *C. A. Angerer* 219

Experiments with *Colpidium campylum* in High-Frequency Electric and Magnetic Fields *Francis M. Summers and Harold K. Hughes* 227

Nutritional Studies of *Paramecium multimicronucleata*. I. Quantitative and Qualitative Standardization of the Food Organism . . . . *Dale Leslie* 243

NUMBER 3

Some Effects of Corpus Luteum Extracts and of Synthetic Progesterone on Young Male Albino Rats . . . . *Jule Kitchell Lamar* 251

Effects of Low Environmental Temperature on the Thyroid and Adrenal Glands of the Ground Squirrel, *Citellus tridecemlineatus* . . . *Moses Zalesky and L. J. Wells* 268

Growth of Protozoan Cultures. II. *Leucophrys patula* and *Glaucoma pyriformis* in a Bacteria-Free Medium . . . . *Morden G. Brown* 277

Biochemical Aspects of the Differentiation of the Female Honeybee (*Apis mellifera L.*) *R. M. Melampy, E. R. Willis, and S. E. McGregor* 283

Paths of Water Exchange in the Earthworm . . . . *A. V. Wolf* 294

Methods for Evaluation of Head Types in Planarians . . . . *William A. Castle* 309

A Migration-Dilution Apparatus for the Sterilization of Protozoa . . *C. Lloyd Claff* 334

The Crustacean Sinus Gland and Chromatophore Activation . *Frank A. Brown, Jr.* 343

The Effect of Concentration of *Colpoda duogenaria* on the Time Required for Encystment in Food-Free Medium . . . . *A. G. R. Strickland* 356

NUMBER 4

The Primary Development of the Skeleton in Nerveless and Poorly Innervated Limb Transplants of Chick Embryos . . . . . *Viktor Hamburger and Molly Waugh* 367

The Stimulus for Maturation and for Ovulation of the Frog's Egg *Francis J. Ryan and Ronald Grant* 383

Physiological Characteristics of the Diapause Grasshopper Egg. I. The Stability of the Diapause Condition . . . . . . *Joseph Hall Bodine and Wilbur A. Robbie* 391

Respiration during Flight in *Drosophila repleta* Wollaston: The Oxygen Consumption Considered in Relation to the Wing-Rate . *Leigh E. Chadwick and Darcy Gilmour* 398

The Respiratory Metabolism of the Frog Retina . . . . . . *Verlus F. Lindeman* 411

The Effect of Thyroid Feeding on Oxygen Consumption of the Goldfish *William Etkin, R. W. Root, and B. P. Mofshin* 415

Nutritional Studies of *Paramecium multimicronucleata*. II. Bacterial Foods *L. D. Leslie* 430

The Effects of Population Density upon Growth, Reproduction, and Survival of *Hyalella azteca* . . . . *Janet Wilder* 439

Index to Volume XIII 463

Physiological Zoölogy

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A PROGRAM OF ANALYSIS AND SYNTHESIS

W. C. ALLEE
Managing Editor of *Physiological Zoölogy*

PROFESSOR C. M. CHILD'S timely article entitled "Lithium and Echinoderm Exogastrulation: With a Review of the Physiological Gradient Concept" inaugurates a series of synthesizing papers in *Physiological Zoölogy* which, it is hoped, will be both extended and useful. Biologists are fairly adequately served at present with publication outlets for detailed scientific analyses. There are also journals devoted to supplying bibliographic citations, abstracts, and reviews, or some combination of these, and a few which specialize in presenting syntheses of scientific material. To our knowledge no journal devoted primarily to publication of the results of biological research is attempting to combine synthesis with analysis and so aid in marking out conceptual trails through the mass of data that is accumulating with great rapidity. It is our purpose to make a beginning toward filling this need.

In earlier days, some traces of which happily remain, the lengthy discussions with which each major scientific paper concluded, attempted to place the detailed material just presented in relation to available knowledge and frequently to develop theoretical treatment for the whole. Now, with the growing trend toward shorter papers, the concluding section, still called a "Discussion," is frequently limited to a restatement in general terms of the main points of the paper (which are again given for the third time in more condensed form in the "Summary") and to a more or less hurried survey of other recent work in the immediate field. Most of us have written such papers and will probably continue to do so.

Under this system long series of clear-cut analytic papers dealing with a developing situation may appear from one or from a number of laboratories, with each paper presenting, under a comprehensive title, one idea, the supporting evidence neatly condensed, and one conclusion, all carefully prepared for ready reference. The title serves as an abstract of the whole, so that, even without more extended abstracting service, the essential contribution need not be overlooked. At times, long series of such detailed reports appear from the same laboratory with no indication of the general ideas, if any, which are being developed as a result of the research. Interested readers can only infer that the author and his associates are working toward some general synthesis.

Ev.K.I.

It is expected that the present series will give a provocative opportunity for presenting three main types of synthesizing papers. In the first place, there will be a chance for a more comprehensive analysis of the many detailed analyses in the world's literature on a given subject; this constitutes one kind of synthesis. In turn, such competent surveys may lead to a synthesis of a higher order, including the development of suggestive theory. In a different category, which is illustrated by Professor Child's paper, we have those cases in which the development of theory has kept step with critical experimentation but in which there is need for a new evaluation of both facts and theory in the light of related developments. Finally, there are those fields in which all we can hope for at present is to have the whole developing situation reviewed by an expert. This will enable us to get the essential facts straight, so that investigation can proceed more intelligently. If skilfully done, these need not result in the obvious hash of the literature characteristic of too many review articles.

It is our conviction that detailed reports of investigations can best be evaluated and interpreted by one who has himself been actively engaged in research work in the field. Such a worker knows the technical pitfalls that may have weakened the supposed conclusions and the essential complexities that must be preserved. For our purposes, we distrust the work of the professional synthesizers. These belong to two or three schools. The first of these can readily be dismissed; their aim is to re-write scientific work so that it can be more readily understood by their public. They often serve a useful function in their own sphere, but our synthesizing series has an entirely different outreach.

Another type of professional synthesizer includes those who, on the basis of training in physical or in philosophical theory, undertake to interpret biological material which they understand imperfectly, in language which can be understood by few others. All too frequently the result is an oversimplification which cannot possibly fit the known facts or one which, despite the triumphant air of the synthesizer, has already been reached by laboratory workers. And while we appreciate verification of our results by others using radically different methods, there is doubtful value in the "discovery," even by a new method, of relations which are already well known.

There is no intention of decrying the aim of theoretical biophysicists whose work is now in its infancy. Perhaps the present attempts at biological synthesis by experimental biologists will furnish a solid and more complete basis for their efforts. Perhaps, too, the philosophers may find here dependable condensations which will be useful in their attempts to unify human knowledge. Unless modern philosophies are based on all phases of our knowledge, including that contributed by modern science, they are clearly inadequate. Equally obviously, the philosopher cannot be expected to master the original documents of science, and he should not be limited to the fields which, for one reason or another, are summarized in book form.

So much for the professional scholars; we have, also, a responsibility to the educated biological public and to students. These, too, deserve to be presented from time to time with readable summaries of the work in active fields of research made by competent research workers who are scholars as well as pioneers.

In order to make a start on this program, the University of Chicago Press has increased its subsidy to enable us to offer at least two synthesizing articles per volume without reducing the budgeted number of pages devoted to publishing the results of original research. The conduct of the analytical part of the journal will continue exactly as in the past, and our pages will remain open to all research papers within our field which meet our standard of excellence. It may be appropriate at this point to emphasize that *Physiological Zoölogy* has no upper limit on the number of pages that will be published in one research article. The relative density of ideas per page and the editors' judgment of the value of the longer article, in comparison with the shorter ones which it replaces, are the criteria used in judging the longer manuscripts that are submitted.

In contrast to the open policy as regards research reports, the series of synthesizing papers will be entirely invitational. The responsibility of the editors will be limited to securing the contribution and to attempting to keep the article to a reasonable length, which we think, in general, should not exceed about fifty manuscript pages, bibliography included. With our present format this will run about twenty-five pages of print.[1]

The following synthesizing articles are in preparation. The exact time and order of their appearance cannot be predicted. Others are being solicited and will be announced later.

E. E. Just, "Unsolved Problems in General Biology."
L. V. Heilbrunn, "The Relation of Protoplasm to the Calcium Ion."
B. H. Willier, "Embryonic Organization in Presomite Chick Blastoderms."
J. H. Bodine, Title to be announced—some phase of cellular physiology.
Alan Boyden, "Serology and Animal Relationship"

[1] A recent analysis gives the following type-load per page for these journals:

	Ems per Page
Journal of Comparative and Cellular Physiology	1,025
Journal of Experimental Zoölogy	1,025
Biological Bulletin	1,310
Physiological Zoölogy	2,010

LITHIUM AND ECHINODERM EXOGASTRULATION: WITH A REVIEW OF THE PHYSIOLOGICAL-GRADIENT CONCEPT

(Eighty-six figures)

C. M. CHILD
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TABLE OF CONTENTS

PAGE
Material and Experimental Procedure 5
Differential and Specific Susceptibilities to External Agents 6
Larval Development of *Dendraster* under Natural Conditions 8
Lithium Modifications with Different Exposure Periods from Early Cleavage 9
Exogastrulation and Entodermization in the Lower Effective Concentrations 11
The Higher Concentrations 13
Lithium Modifications with Different Exposure Periods Beginning at Later Stages . . . 20
Variations in Susceptibility in *Dendraster* 23
Entodermization by Other Agents 23
Discussion:
- Inhibitory Ectodermal Modifications 26
- Inhibitory Modifications of Prospective Entoderm 27
- Entodermization of Prospective Ectoderm 28
- Mesenchyme 29
- Secondary Modifications 30
- Reconstitution in Exogastrulae 32

The Physiological-Gradient Concept 34
Summary 40
Literature Cited 41

IN EARLIER papers gradient patterns of normal sea-urchin and starfish development and their alterations in exogastrulae, as indicated by differential dye reduction in low oxygen, were described, and their relations to the primary pattern of echinoderm development were discussed (Child, 1936*a*, 1936*b*). The present paper is a further experimental study of exogastrulation and related modifications in the echinoid, *Dendraster excentricus*, with particular reference to their bearing on the general problem of developmental pattern. To this is appended a review of the physiological-gradient concept.

The experiments of Herbst made it evident that lithium is highly effective in producing exogastrulation, but it has also been shown that many other physical and chemical agents are also more or less effective. It is still maintained by some, however, that the action of lithium as an exogastrulating agent is in some way unique and indicative of regional localization or graded distribution of specific substances. For example, Runnström and his co-workers postulate two opposed and overlapping concentration gradients of specifically different substances—an "animal" and a "vegetative" (vegetal) gradient in the sea-urchin egg—and maintain that lithium favors, increases, and extends the vegetal gradient, supposedly by favoring formation of the vegetal substance.[1] Apparently this is regarded as a specific chemical effect. It was pointed out in the paper cited above that it is difficult to interpret certain experimental data in terms of this hypothesis, for lithium does not always or necessarily increase entoderm at the expense of ectoderm but, under certain conditions, inhibits entoderm more than ectoderm. The method most frequently used to produce exogastrulation consists in exposure for a number of hours from early developmental stages to certain ranges of concentration of LiCl, the effective range differing with different species, followed by return to water. With this procedure exogastrulation may or may not occur before return to water, according to concentration of LiCl and exposure period. Certain ranges of concentration and exposure periods produce merely differential inhibition of early development, and exogastrulation occurs after return to water. With other concentrations and exposure periods exogastrulation may occur in the solution, but great changes in relative proportions of parts may follow on return to water, and development may proceed much farther than with continuous exposure to lithium. Also, other effects of continuous exposure may be very different from those of temporary exposure.

Interpretations of some of these effects have been suggested (Child, 1936*b*); but in general the question of their physiological significance has received comparatively little attention, and the possibility of secondary modifications resulting from differential tolerance, conditioning, or recovery has usually been ignored. Since this is the case, it may still be questioned whether or to what extent lithium exogastrulae of echinoderms depend on specific regional features of developmental pattern rather than on nonspecific regional differentials or gradients involving quantitative dynamic factors and differential susceptibility. It is believed that the experimental data presented in this paper throw some further light on this question.

To the director and the staff of the Hopkins Marine Laboratory I am again deeply indebted for their kindness in providing facilities and material, making these further experiments possible during the summer of 1938.

MATERIAL AND EXPERIMENTAL PROCEDURE

In the experiments of 1938 the sand dollar, *D. excentricus*, served exclusively as material; in earlier experiments *Strongylocentrotus purpuratus*, *S. franciscanus*, *Arbacia punctulata*, *Echinarachnius parma*, and the asteroids, *Patiria miniata* and *Asterias forbesii*, had been used. The 1938 experiments consisted primarily in a systematic study of effects of LiCl from the lowest concentrations producing any appreciable modification of development to those completely inhibitory and lethal, that is, fifteen concentrations from m/120 to m/10. Most of these concentrations were used both in approximately isotonic and in somewhat hypertonic sea-water solutions, but the only difference observed was that in the higher concentrations the hypertonic solutions were somewhat more effective in producing exogastrulation and other modifications. All concentrations given below are approximately isotonic unless otherwise stated. In addition to the wide range of concentrations, a wide range of exposure periods was also used: from 1–2 hours to continuous from first cleavage; and exposure was begun at different stages: first cleavage, sixteen to thirty-two cells, early, middle, and late blastulae, mesenchyme immigration, beginning and mid-gastrulation. Experimental lots, some one hundred and fifty in number, were examined at frequent intervals in order to determine the course of developmental modification as well as the forms finally attained. These experiments, performed at the height of the *Dendraster* breeding season, showed a somewhat lower susceptibility of corresponding stages to LiCl than those of 1935, which were on material at the extreme end of the breeding season, when ripe gonads were still present in less than 1 per cent of animals collected. With that material greater or less degrees of exogastrulation occurred rather frequently in water, but only very rarely in a few individuals in the material of 1938. In lithium experiments of 1935 with *Patiria* as material, m/10, m/20, m/25, and m/30 were used on many lots with exposure beginning at different stages and for different periods. Earlier experiments with *Arbacia*, *Echinarachnius*, and *Asterias* were chiefly concerned with a few concentrations found to produce exogastrulation; but the results obtained, though fragmentary, were essentially similar to those of recent work as regards developmental modifications. Some data on exogastrulation by other agents have also been obtained.

---

[1] See Lindahl, 1936, and Runnström's papers cited by him.

Except in a few experiments on crowding, development took place in approximately 250 cc. of solution or water in practically flat-bottomed finger bowls set in flowing sea water and glass-covered in diffuse daylight. Care was taken to avoid crowding, and aggregations were often dispersed by gentle stirring. Approximate percentages of exogastrulae were noted with most lots, also high or low frequency of other modifications; but the chief purpose of the experiments was the study of the characteristic features of developmental modifications of individuals rather than their frequencies.

Figures are semidiagrammatic but based on ocular micrometer measurements of the various dimensions of living individuals, immobilized if necessary. The measurements were supplemented by many hundred sketches. General distribution of mesenchyme and of cells given off from the entoderm is approximately indicated in diagrammatic manner. Position and general pattern of skeleton is shown essentially as recorded in the sketches. Skeletons of anal arms are represented as single rods, though usually consisting of two apposed rods with cross-connections and in modified forms sometimes of three or four rods. Pigment is not indicated.

DIFFERENTIAL AND SPECIFIC SUSCEPTIBILITIES TO EXTERNAL AGENTS

The question whether echinoderm exogastrulation represents a specific regional action of external agents was discussed earlier (Child, 1936*b*). It was pointed out that, since exogastrulation is produced by many agents,[2] it cannot be regarded as a specific effect of any particular agent. Some agents are more effective than others, and lithium appears to be particularly effective. The high effectiveness of lithium is doubtless associated in some manner with its point of attack on living protoplasms, but this does not necessarily mean that its effect is on a regionally localized specific substance or substance-complex. The question of differential susceptibility of organisms to external agents has been repeatedly discussed,[3] but it seems necessary to call attention again to certain points as a basis for presentation of experimental data.

---

[2] It is not necessary to repeat the list of agents given in the earlier paper, but nickel chloride is to be added (Waterman, 1937); and the occasional occurrence of a few exogastrulae with di-nitro-compounds, iodoacetic acid, and pyocyanine is reported (Waterman, 1938).

[3] Child, 1928, and references given there to earlier work on differential susceptibility. In the earlier paper on exogastrulation it was briefly discussed with particular reference to echinoderm development (Child, 1936*b*, pp. 458–61).

It has often been pointed out that susceptibilities of early developmental stages of various animals and of the adults of many of the simpler organisms show a gradation or differential in the same direction in relation to axiate pattern with many chemical and physical agents and conditions in certain ranges of concentration or intensity. This differential susceptibility does not give evidence of specific substances regionally localized along a physiological axis with corresponding specifically different regional effects of different external agents. It suggests, rather, that axiate pattern is primarily a quantitative gradation or gradient in physiological condition. If this is granted, it appears beyond question that regionally localized specific differentiations, chemical and morphological, originate within this gradient pattern; when a certain degree of such differentiation is attained, specific differences in susceptibility begin to appear, and with further progress of differentiation become more distinct.

Differential susceptibility appears in various aspects. In direct differential lethal action and in differential inhibition of development a certain parallelism appears between susceptibility and rate of physiological activity, as indicated by rate of respiration, rate of dye reduction in low oxygen, and in many cases by rate of developmental activities. Regions most intensely active are most susceptible to direct lethal and inhibitory action, and the susceptibility gradient is, in general, parallel to the physiological gradient. With certain lower ranges of concentration or intensity of external agents differential tolerance, differential acclimation or conditioning, and, after temporary exposure, differential recovery may occur as secondary effects. In these secondary effects the regions most susceptible to the higher, directly lethal, or inhibitory ranges of concentration or intensity show, in general, the greatest tolerance, become acclimated or conditioned with continued exposure to the agent, or recover after temporary exposure most rapidly or completely. Tolerance, conditioning, and recovery decrease along an axis in the same direction as physiological activity. With certain ranges of action of agents, partial conditioning or recovery may occur; that is, a part of the organism may be killed or so inhibited that secondary changes are impossible while a less susceptible part may live long enough to become more or less conditioned or may recover on removal from the agent. These partial secondary effects are, as might be expected, most conspicuous in recovery. More susceptible regions may be killed, and the rest of the organism may recover after removal from the agent.

The susceptibility gradients are not directly related to the particular ways in which different agents interfere with the protoplasmic system. It appears evident that any sort of interference sufficient in degree will be more rapidly or more intensely effective in regions of greater, than in those of less, physiological activity, and that the more active regions will, in general, show greater tolerance or become conditioned to, or recover more rapidly or completely from, slight interference than the less active regions. Perhaps tolerance, conditioning, and recovery are not possible with some agents; but it is certain that they do occur with many.

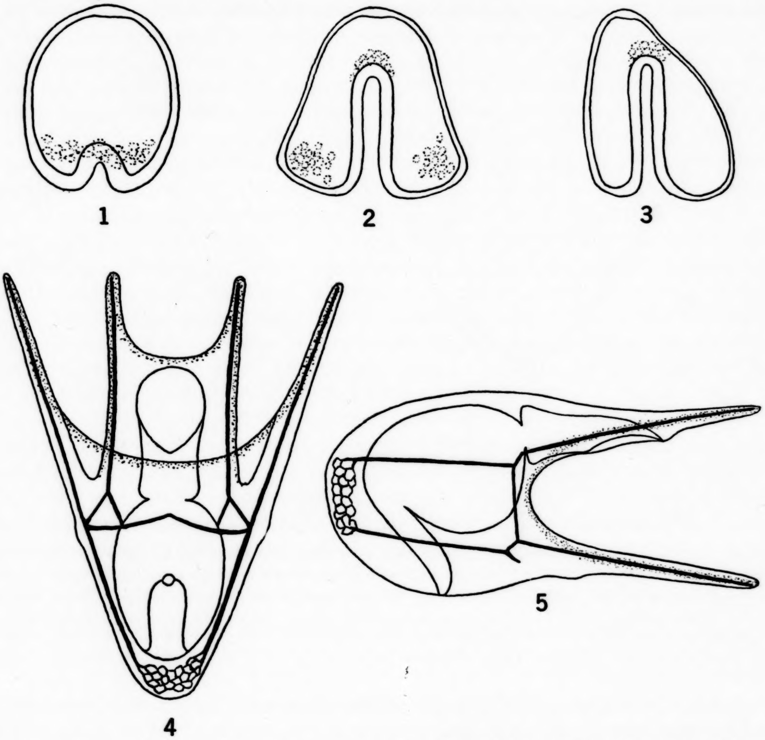
These various expressions of differential susceptibility make possible differential modification of development in two opposite directions. In differential inhibition the "high" regions of the physiological gradients are most inhibited, most reduced in size, or may even be killed. In differential tolerance, conditioning, and recovery the high regions of the gradients, though primarily most inhibited, show secondarily the greatest development.

The absence of regional specific susceptibilities along a physiological axis does not necessarily mean that qualitative or specific differences of substance are absent in that axis. At most, it can mean only that such differences are not sufficient to become a basis for specific differences in susceptibility. In as complex a system as a living protoplasm, the many differences in concentration of a multitude of substances and in condition of the colloid substrate, associated with or resulting from the differences in rate of metabolism at different levels of a gradient originating as a gradient in rate, provide ample basis for the origin of regional qualitative differences. These may begin to appear from the moment of establishment of a quantitative physiological gradient, but quantitative factors may remain predominant for a shorter or longer time. Probably so-called "determination" of a part is associated with attainment of a certain degree of specific or qualitative character by that part of a gradient pattern. The following experimental data bear upon the question whether or to what extent such localizations are primarily concerned in exogastrulation and related modifications of early development.

LARVAL DEVELOPMENT OF *Dendraster* UNDER NATURAL CONDITIONS

The early gastrula, the later gastrula, viewed laterally and ventrally, and the pluteus in anal and lateral view are shown in Figures 1–5. It will be noted in Figure 3 that the ventral side of the advanced gastrula and prepluteus becomes almost flat and that the tip of the archenteron approaches the dorsal wall before it bends ventrally toward the stomodeal region. The oral lobe of the pluteus develops from the apical region ("animal" region), as in other echinoids. It is proportionally considerably larger than the oral lobes of *Arbacia* and *Strongylocentrotus*. The short oral arms are parallel or almost parallel, and the angle between the anal arms (brachial angle) does not vary greatly. These figures serve as a basis for comparison with the alterations of form and proportions under experimental conditions.

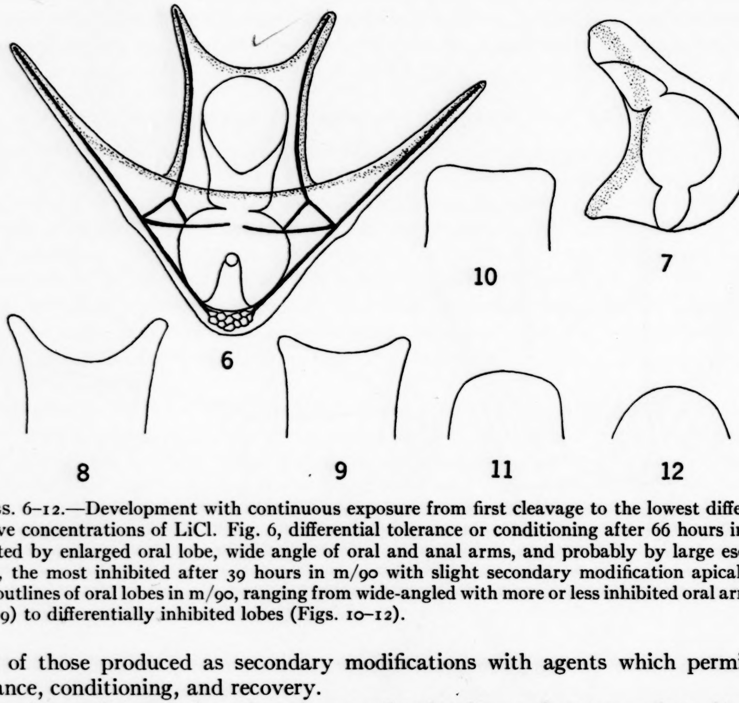
Differential reduction of dyes in low oxygen (Child, 1936*a*) and differential death in various agents indicate that the apical region is in early stages the high end; the basal ("vegetal") region, the low end of the primary apicobasal gradient; and that, as gastrulation approaches, the basal region—mesenchyme and entoderm—undergo a change in condition, apparently a rather intense activation of some kind, indicated by marked increase in rate of dye reduction and in lethal susceptibility. Development of the oral lobe from the apical region apparently involves a secondary activation there in later gastrula stages, and each developing anal arm is a region of increased rate of dye reduction and increased susceptibility. In earlier blastula stages no difference in rate of dye reduction or susceptibility, indicating ventrodorsality, has been observed. In the more advanced blastula, still radial in form, rate of dye reduction and susceptibility become distinctly higher on one side. That this is the ventral side, distinguishable physiologically before it is morphologically, is highly probable from the fact that the ventral side, as soon as directly distinguishable, reduces dye more rapidly and is more susceptible than the dorsal. According to these observations, the ventral side appears at first to be merely the higher levels of a ventrodorsal gradient; but later the stomodeal region and anal arm primordia become distinguishable as regions of higher rate of dye reduction than the rest of the ventral side, the rate decreasing from a center in each. The gradual appearance of the ventrodorsal gradient in the course of pregastrular development, as indicated by the methods employed, is a point of some interest in relation to experimental modifications. For example, with differential inhibition beginning in early stages, ventrodorsality may never develop at all; but inhibition beginning in later blastula or in gastrula stages usually merely decreases its developmental expression. During and immediately after immigration of mesenchyme, the rate of dye reduction becomes more rapid in the mesenchyme than in any other part, and more rapid in entoderm than in ectoderm; also, the invaginating entoderm develops a dye reduction gradient with high end at the tip, and experiments indicate a parallel differential susceptibility. In the fully developed pluteus the various developmental gradients disappear or become less distinct, but nothing is known concerning origin of new gradient systems in metamorphosis.



FIGS. 1–5—Larval development of *D. excentricus* under natural conditions. Fig. 1, early gastrula; Figs. 2 and 3, ventral and lateral outlines of later gastrula; Figs. 4 and 5, pluteus in anal and lateral view.

LITHIUM MODIFICATIONS WITH DIFFERENT EXPOSURE PERIODS FROM EARLY CLEAVAGE

Concentrations of LiCl below m/90 or m/80 rarely produce exogastrulae but do modify development differentially. The forms produced are of interest in comparison with certain characteristics of exogastrulae and require brief notice. Continuous exposure from first or early cleavage to m/120 produces characteristically plutei of the type of Figure 6 with large oral lobe, diverging oral arms, brachial angle wider than normal, and large mouth and foregut (cf. Figs. 4 and 6). Development is primarily retarded, as compared with controls; and the pluteus form is a secondary modification, resulting from greater tolerance or conditioning to lithium of the higher levels of the apicobasal and ventrodorsal gradients and probably also of the high end of the archenteric gradient. Development in m/110 and m/100 usually produces forms of the same type, except that oral arms are shorter or absent and that the oral lobe is merely rounded in many individuals; that is, more inhibition and less secondary modification of this region occurs. Anal arms are usually also somewhat shorter, and the angle less wide. All these forms are essentially similar to the wide-angled plutei of *Arbacia* developing in differential tolerance, conditioning, and recovery with various agents, though less extreme than some of those produced as secondary modifications with agents which permit early tolerance, conditioning, and recovery.



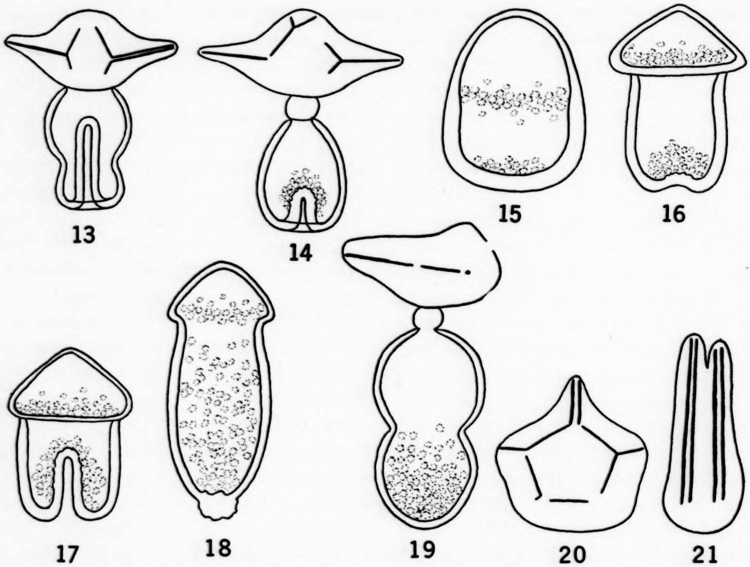
Figs. 6–12.—Development with continuous exposure from first cleavage to the lowest differentially effective concentrations of LiCl. Fig. 6, differential tolerance or conditioning after 66 hours in m/120, indicated by enlarged oral lobe, wide angle of oral and anal arms, and probably by large esophagus; Fig. 7, the most inhibited after 39 hours in m/90 with slight secondary modification apically; Figs. 8–12, outlines of oral lobes in m/90, ranging from wide-angled with more or less inhibited oral arms (Figs. 8 and 9) to differentially inhibited lobes (Figs. 10–12).

In m/90 continuously from first cleavage the plutei range from somewhat wide-angled forms with large oral lobe, but with oral arms more or less inhibited and anal arms shorter than normal, to forms with only slight secondary modification of the apical region and very short or no anal arms (Fig. 7). Figures 8–12 show outlines of oral lobes of these forms, ranging from wide angled, but with oral arms somewhat inhibited (Figs. 8 and 9), through various degrees of differential inhibition (Figs. 10–12).

With continuous exposure to m/80 there is no certain evidence of secondary modification. The plutei are smaller than controls; the oral lobe is differentially inhibited and without oral arms; the anal arms are only about half the control length; and the relative size of entodermal divisions differs little from normal. Animals returned from all these low concentrations to water a few hours before gastrulation develop a high percentage of plutei like controls; but a few slightly wide-angled forms with large oral lobe may appear, indicating slight degrees of differential recovery.

EXOGASTRULATION AND ENTODERMIZATION IN THE LOWER EFFECTIVE CONCENTRATIONS

With sufficiently long or continous exposure from first or second cleavage to m/70 or m/60 the forms commonly known as exogastrulae appear in percentages ranging from 10 to 50 in different lots. Most of these, however, are entexogastrulae; examinations during their development show that the original entoderm is completely or partially invaginated—but is inhibited in further development, and additional entoderm, which shows no tendency to invaginate, originates from entodermization of prospective ectoderm by lithium. With these concentrations entodermization involves only the more basal levels of prospective ectoderm (Figs. 13 and 14). Certain features of these forms require attention. First, lithium not only transforms more or less of the prospective ectoderm into entoderm but also inhibits development of the original prospective entoderm. The invaginated entoderm shows no differentiation into the usual three regions, ends free in the blastocoel, and in Figure 14 is giving off cells additional to the primary mesenchyme, an effect of inhibiting conditions, as will be more clearly evident below. Second, the entodermized ectoderm which forms most or all of the external entoderm of these forms remains in essentially the same relation to the rest of the individual as when it was prospective ectoderm. It has become entoderm but has not evaginated, instead of invaginating; and it has not changed its position. Are, then, forms like Figures 13 and 14, strictly speaking, exogastrulae? This question arises as regards many other so-called exogastrulae and will be discussed later. Meanwhile the terms "exogastrulation" and "exogastrula" will serve for descriptive purposes as indicating the condition or individual with noninvaginated entoderm, but without implication as to origin of the entoderm. Exogastrulation of the original prospective entoderm does occur in many individuals of the same lots as Figures 13 and 14; it may be partly evaginated in Figure 14. However, as will appear, there are many forms with noninvaginated entoderm in which the original prospective entoderm is only in small part or not at all involved. Third, development of the entodermized ectoderm is inhibited in lithium; with continuous exposure it does not develop far beyond the condition shown in Figures 13 and 14. Fourth, the apical region of the remaining ectoderm in these forms is greatly inhibited, but anal arms develop at a wide angle. Conditions determining these modifications, however, are quite different from those which result in wide-angled plutei like Figure 6. It was noted above that the ventral side of the advanced gastrula is flat (Fig. 3). In forms like Figures 13 and 14 and in other similar forms described and figured below, further development of the ventral side is inhibited to such a degree that it remains almost flat or may, under certain conditions, become convex instead of deeply concave, as in normal plutei (Figs. 1 and 2). Moreover, the ectoderm of Figures 13 and 14 is only the apical half more or less of the prospective entoderm. Development of this region involves extensive reconstitution and decrease in scale of organization. Arms develop from ectoderm which, under natural conditions, would give rise to parts intermediate between arms and oral lobe or to proximal parts of the lobe. Arm development is localized at the lateral borders of the ventral side of the region retaining ectodermal character; but since this remains more or less flattened or becomes convex, arms grow out at a wide angle, conditions on the ventral side apparently being the chief factors in determining direction of their growth, perhaps by determining direction of growth of skeletal rods. According to this interpretation, forms of this general type represent primarily differential inhibitions. Development of prospective entoderm, entodermized ectoderm, and remaining ectoderm is inhibited; in the remaining ectoderm apical and ventral regions are most inhibited. However, activation and outgrowth of arm tissues, a feature of relatively late stages, and perhaps also some relative increase of ventral area, may represent some degree of differential conditioning in the cases of Figures 13 and 14 or of recovery in various other similar forms (see Figs. 32, 33, and 46); but development of the oral lobe from the apical region, primarily the most susceptible region of the embryo, is completely inhibited. It is also possible that outgrowth of arms is determined by the skeletal rods. In any case, secondary modification in these forms is slight, if present at all; they differ greatly from the secondary modifications after slight inhibition, such as Figure 6.



FIGS. 13–21.—Modifications by m/70, m/60, and m/50. Figs. 13 and 14, modifications occurring in percentages from 10 to 50 with continuous exposure from first cleavage to m/70 or m/60. Fig. 15, 15–18 hours in m/50 from two-cell stage, entodermization of basal prospective ectoderm, dissociation of cells from original prospective entoderm; Figs. 16–18, most advanced stages attained in m/50; Figs. 19–21, development following return to water from m/50 at stage of Fig. 15; Fig. 20, ectoderm of Fig. 19; Fig. 21, another ectoderm with fused arms.

With continuous exposure to m/70 or m/60, beginning with the 5-hour blastula, plutei with slightly inhibited oral lobe and anal arms result. These concentrations are not high enough to produce exogastrulation or entodermization from these stages on, but higher concentrations are still effective at this stage. Evidently "determination" of parts has become somewhat more stable during 5 hours of development. With return to water from m/70 or m/60 before development is too far advanced, some evidence of differential recovery appears, but higher concentrations provide more interesting examples.

THE HIGHER CONCENTRATIONS

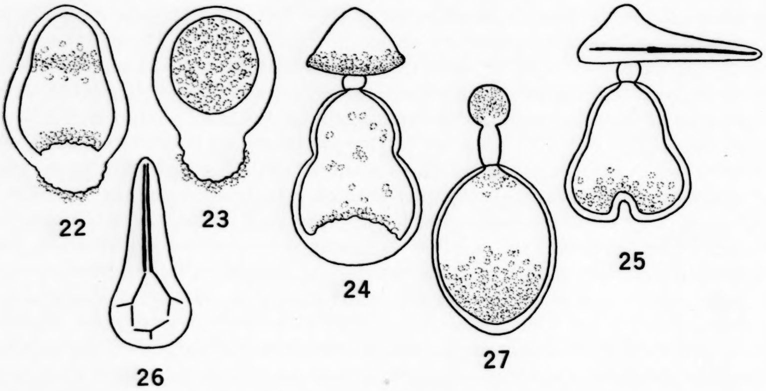
Concentrations of m/50 and above are highly effective in inhibiting development in *Dendraster;* and with certain exposure periods at early stages, effective in producing exogastrulation. The usual condition after 15–18 hours in m/50 from two-cell stage is shown in Figure 15. The circle of primary mesenchyme, which immigrates to a level slightly apical to the ecto-entodermal boundary, is in these forms about midway between the poles; that is, the basal half, more or less, of the prospective ectoderm has become entoderm. In a few individuals some invagination of prospective entoderm has taken place, but in all there is dissociation of cells from it. The most advanced stages attained with continuous exposure are shown in Figures 16–18. The ectoderm remains radial; the primary mesenchyme remains, in large part, in a circle and does not form skeleton; in many individuals the prospective entoderm continues to give off cells into the blastocoel or may lose more or less completely its epithelial character and become a solid cell mass (Fig. 18). Evidently its further development is inhibited, even when it invaginates (Fig. 17). Following return to water at the stage of Figure 15 the entodermized ectoderm elongates and usually differentiates into two regions (Fig. 19). The small "neck" connecting ectoderm and entoderm appears very commonly in exogastrulae as a secondary modification; its possible significance is discussed later. The remaining ectoderm recovers sufficiently to develop a differentially inhibited ventrodorsality with a single arm (Figs. 19 and 20), fused arms (Fig. 21), arms diverging at subnormal angle, and a few wide-angled individuals with inhibited oral lobes. Further development of entodermized ectoderm also results from recovery, but the original entoderm gives no definite evidence of recovery and may be largely dissociated into free cells in the blastocoel.

Figure 22 shows the characteristic condition after 18 hours in m/35; extremes under these conditions are like Figures 15 and 23. In Figure 22 entodermization extends farther apically than in Figure 15, and the original entoderm has lost its epithelial order and is undergoing dissociation. In Figure 23 most or all of the original entoderm is a solid mass of cells dissociating externally but has probably also given off cells into the blastocoel. Development does not proceed much farther in this concentration. Some enlargement and decrease in thickness of the cell wall may occur; the original entoderm apparently undergoes complete dissociation; sooner or later the entodermized ectoderm begins to dissociate; and the form of the whole gradually becomes spherical and remains so until death.

In lots returned to water at the stage of Figures 22 and 23 the characteristic form is of the type of Figure 24, with a radial ectoderm, a circular transverse ciliated band about its basal region, the original entoderm a more or less dissociated cell mass without pattern, and entodermized ectoderm large, with two regions separated by a constriction. At one extreme a few individuals develop a one-armed ectoderm, and one case of partial invagination was recorded (Figs. 25 and 26). At the other extreme are almost completely entodermized forms with original entoderm largely or wholly dissociated and with little or no enlargement of entodermized ectoderm, that is, with little or no evidence of recovery (Fig. 27).

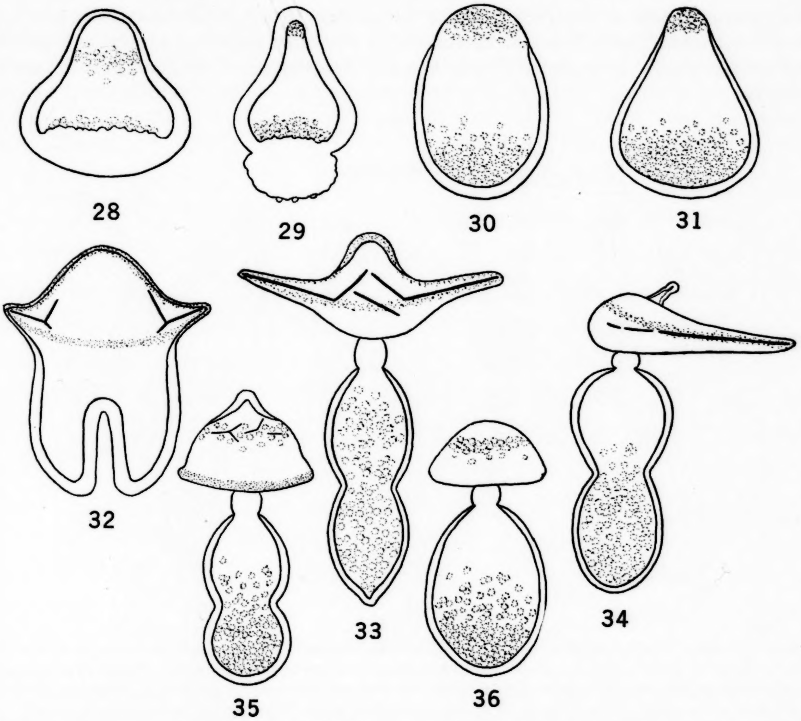
With certain exposure periods m/30 and m/25 produce 100 per cent exogastrulae. A number of series were run in these concentrations with continuous exposure and with return to water after different exposure periods of portions of the same lot. After 23 hours in m/30 from first cleavage the original entoderm is a cell mass undergoing dissociation either in the blastocoel or outside, and prospective ectoderm is in large part or almost wholly entodermized (Figs. 28 and 29). The condition of those still alive after 47 hours in m/30 is shown in Figures 30 and 31. Approach to spherical form, dissociation of cells from the remaining epithelial wall, and death result from longer exposure.

Animals from the same lot returned to water after 3 hours become entogastrulae and develop into normal plutei. In water after 5 hours' exposure the original prospective entoderm invaginates, entodermization of about the basal half of the prospective ectoderm occurs, entexogastrulae result, and the remaining ectoderm develops ventrodorsality (Fig. 32) and finally approaches pluteus form with inhibited oral lobe and short arms at varying wide angles. In a few individuals a single apical coelom, apparently without bilaterality, develops from the invaginated entoderm, as in Figure 45, evidently a differential inhibition of this region; but no further regional differentiation of entoderm appears. In water after 8½ hours' exposure, there is no invagination of entoderm (Figs. 33–35). Fifteen hours after the return of this lot to water the original entoderm was wholly or almost wholly dissociated. Some of its cells perhaps form a part of the terminal entodermal region of these forms, but most of them are free in the blastocoel or lost externally, and the entodermal epithelium is largely or wholly entodermized ectoderm. Its large size (Figs. 33–35) results not from a direct effect of lithium but from differential recovery after return to water. Ectodermal development ranges from forms with short, wide-angled arms through one-armed forms to completely radial. The secondary thickening of the apical region in some individuals (Figs. 33 and 35) indicates slight differential recovery. Mesenchyme in the radial forms lies near the apical region, and a circle of spicules often develops there (Fig. 35). Since the circle of mesenchyme is near the ecto-



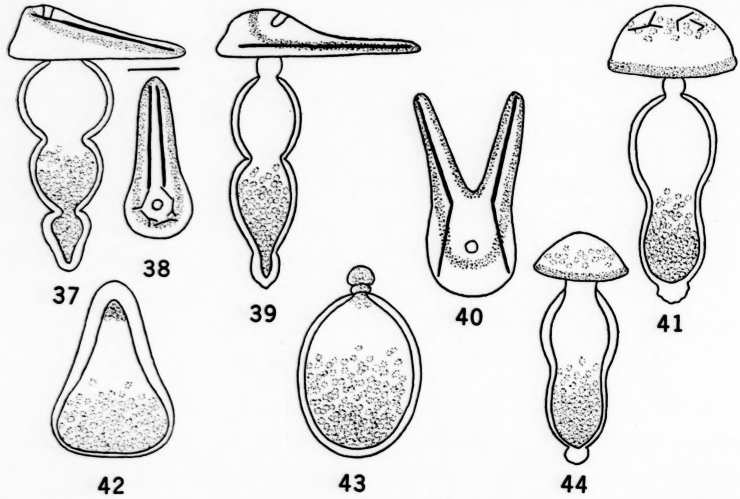
FIGS. 22–27.—Effects of m/35 LiCl. Figs. 22 and 23, 18 hours in m/35 from first cleavage; Fig. 24, the characteristic form developing following return to water after 18 hours' exposure; Figs. 25–27, extreme forms of same lot.

entodermal boundary in earlier stages (Figs. 15, 22, 28, and 29), its position in these forms suggests that there may have been a re-ectodermization of entodermized ectoderm after return to water. Occasionally an evaginated, relatively large stomodeum develops with indication of two more or less distinct regions (Fig. 34). The evagination may be a lithium effect, but the large size is undoubtedly a result of differential recovery. In water after 10 hours' exposure to m/30 there is less recovery, less enlargement of entodermized ectoderm, and the remaining ectoderm is always radial with circle of mesenchyme or a few spicules near its apical pole, again suggesting some re-ectodermization (Fig. 36). When a ciliated band develops on these ectoderms, it is a transverse circle at the margin of the flat basal region of the ectoderm.



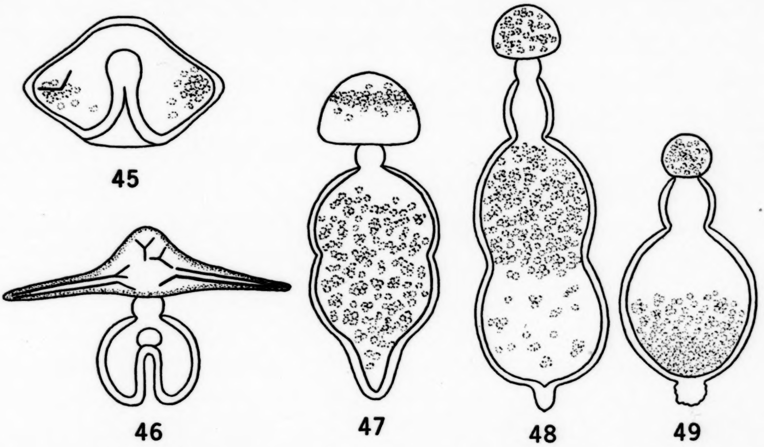
Figs. 28–36.—Modifications of single lot of eggs with different exposure periods to m/30 from first cleavage. Figs. 28 and 29, continuous, 23 hours; Figs. 30 and 31, continuous, 47 hours; Fig. 32, 22 hours in water after 5 hours in LiCl, further approach of ectoderm to pluteus form in later stages, not figured; Figs. 33–35, forms developing in water after $8\frac{1}{2}$ hours in m/30, with evidence of differential recovery of apical region in Figs. 33 and 35, of entodermized ectoderm in all, and in development of everted stomodeum in Fig. 34; Fig. 36, development in water after 10 hours in m/30.

A few forms from other lots of eggs in m/30 are shown in Figures 37–44. Figures 37–41 are forms developing in water after $18\frac{1}{2}$ hours' exposure from the eight- to the sixteen-cell stage. They show somewhat more development of entodermized ectoderm than Figures 35 and 36, although longer in LiCl. In Figures 37, 39, and 41 the terminal region of the entoderm is probably a part of the original prospective entoderm. In Figure 41 the skeletal spicules are in the apical region; in many individuals of the lot spicules or mesenchyme cells are in this position, indicating probable re-ectodermization after return to water. Figures 42–44 from another lot show the condition after 27 hours in m/30 from first cleavage (Fig. 42) and the range of forms developing after return to water at this time (Figs. 43 and 44). The thin epithelium in the basal region of Figure 42 seems to be part of the original entoderm which has not dissociated. This condition appears frequently.



FIGS. 37–44.—Other examples of modification with different exposure periods to m/30. Figs. 37 and 39, lateral, and Figs. 38 and 40, apical, views of forms developing in water after 18½ hours in m/30 from the eight- to the sixteen-cell stage; Fig. 41, radial form from same lot; Figs. 42–44, return to water after 27 hours in m/30 (Fig. 42) and range of forms developing later (Figs. 43 and 44).

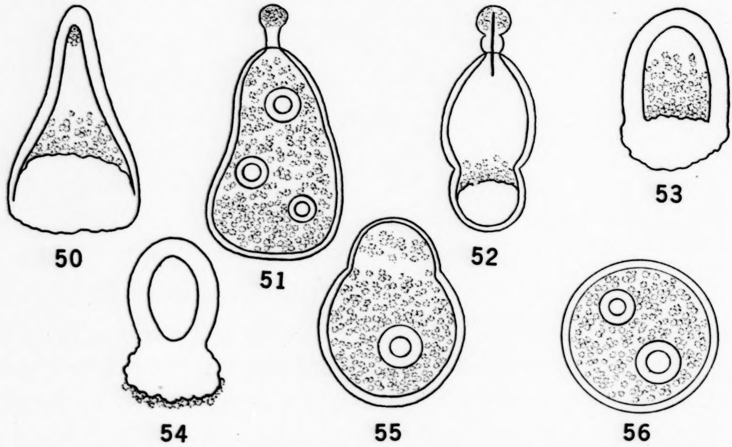
Series with different exposure periods to m/25 from first cleavage present some further points of interest. With development in water after 3 hours' exposure, entoderm invaginates in all, and only about 10 per cent show any entodermization of prospective ectoderm; but after 22 hours in water, differential inhibition is still evident: the apical ectodermal region is inhibited, oral lobe is absent, and the apical entoderm is often a solid cell mass (Fig. 45); but these forms gradually undergo more or less differential recovery and may become small plutei with inhibited oral lobe and short arms. In water after 5 hours' exposure invagination of entoderm often occurs, but there is usually more or less dissociation, with or without invagination. In Figure 46 the most highly developed individual observed in the lot, an apical coelom with no evidence of bilaterality, develops from the invaginated entoderm. Like the similar case mentioned above, this results from a mediolateral differential inhibition essentially like that producing approximated, fused, and single anal arms in echinoid development and approximation of bilateral cephalic organs and cyclopia in planarians and in vertebrates. The characteristic form of this lot is Figure 47 with radial ectoderm, subapical circle of mesenchyme, large entodermized ectoderm, and more or less complete dissociation of original entoderm. In water after 7½ hours' exposure, all individuals become essentially like Figure 47, except that the ectoderm averages somewhat smaller. In all these the mesenchyme is subapical, as in Figure 47, probably in consequence of re-ectodermization during recovery. The characteristic condition after 21½ hours' exposure is very similar to that shown in Figure 42, except that the original entoderm is still largely a solid mass. Figures 48 and 49 show the most advanced development after return to water at this time: in these the cell mass at the tip of the entodermized ectoderm probably represents part of the original entoderm.



FIGS. 45–49.—Modifications by m/25 with various exposure periods from first cleavage. Fig. 45, 3 hours in m/25, 22 hours in water, differential inhibition decreasing basipetally in ectoderm and in archenteron; Fig. 46, most highly developed larva in water after 5 hours in m/25; Fig. 47, the characteristic form developing in water after 5 hours in m/25; Figs. 48 and 49, characteristic development in water after 21½ hours in m/25.

It is a point of some importance that with all the shorter periods of exposure to m/30 and m/25 (less than 10 hours) the animals at the time of return to water are, in any case, only slightly different from the controls. Even with the longer of these exposures there is only a slight differential inhibition decreasing from the apical region basipetally indicated by smaller size of the apical region than in controls and retardation of the decrease in thickness of the blastula wall. All further modifications of development occur after return to water and evidently represent combinations of the direct inhibiting effect of lithium and the secondary modifications associated with recovery from that effect. The lithium effect is progressive, and recovery from it is less rapid and less complete the longer the exposure from any given stage of development.

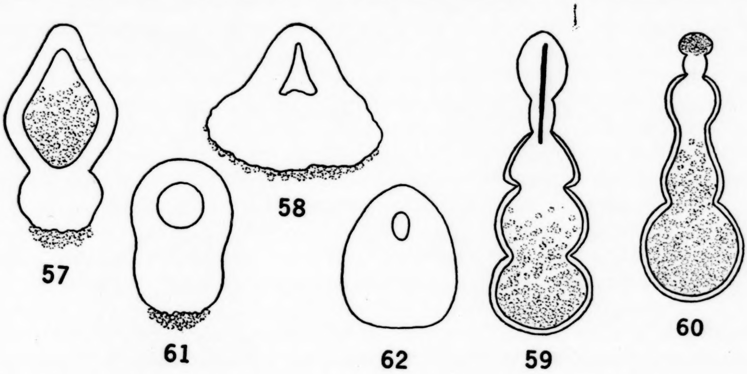
Even with m/20 certain results of interest appear. In approximately isotonic m/20 only large-celled early blastula stages are attained after 9 hours; controls are advanced blastulae. With return to water at this time, characteristic forms 17 hours later are like Figures 42 and 50. The whole basal region has lost epithelial character and is more or less completely dissociated; in some individuals pigment appears in the extreme apical region. These forms stick together in large numbers by their basal regions, with the more apical epithelial portions protruding from the surface of the mass, and gradually become large, irregular masses full of dissociated cells; dissociation may finally become complete except for an external epithelium surrounding the multiple mass. All the individuals involved in one of these masses have lost all individuality, and most of their cells have become free cells in the interior of the mass; only a surface-interior pattern remains, and death finally occurs without further change. Forms like Figure 50, if they remain single, may develop, as shown in Figures 51 and 52. In many of them one or more spherical epithelial vesicles form free in the blastocoel from some of the entoderm cells (Fig. 51). Similar epithelial vesicles often appear in considerable numbers in the large multiple masses if they are returned to water early enough. Single individuals occasionally develop a single skeletal rod extending apicobasally (Fig. 52). After 26 hours in m/20 entodermization of ectoderm is apparently complete; and dissociation, either into the blastocoel or externally or both, is progressing acropetally from the basal pole (Figs. 53 and 54). In some individuals cytolysis is beginning in the apical region. Many are still within the fertilization membrane. After return to water at this stage only 20–25 per cent remain alive for 24 hours, but these show some evidence of recovery. In some of them more or less ectoderm develops (Fig. 55), although the earlier stages show no visible evidence of any remaining ectoderm (Figs. 53 and 54). Re-ectodermization seems to have occurred in these cases, as in so many others. Other individuals are



FIGS. 50–56.—Modifications with different exposure periods from the first cleavage to m/20. Fig. 50, 9 hours in m/20, 17 hours in water; Figs. 51 and 52, 9 hours in m/20, 40 hours in water, entodermal epithelial vesicles in Fig. 51, a single skeletal rod in Fig. 52. Figs. 53 and 54, 26 hours in m/20; Figs. 55 and 56, 26 hours in m/20, 24 hours in water, apparently some re-ectodermization in Fig. 55, with Fig. 56 probably all entoderm.

spherical, apparently completely anaxiate with thin surface epithelium probably consisting entirely of entodermized ectoderm (Fig. 56), though it is not possible to determine whether it is to be regarded as entoderm or as re-ectodermized. One to several epithelial vesicles are present in almost all (Figs. 55 and 56), and many of these vesicles from disintegrated individuals persist free in the water. The reappearance of epithelial character in entodermal cells is evidently a result of recovery. Whether it represents cell aggregation or results from cell division has not been determined.

A sea-water solution of LiCl m/20 is, of course, highly hypertonic, and its effects are somewhat more extreme in certain respects than those of approximately isotonic solution. The condition after 7 hours in this solution from first cleavage and 12 hours in water is shown in Figures 57 and 58. Formation of a solid cell mass progressing acropetally from the basal region, usually with more or less dissociation of cells from its surface, has occurred to a greater or less degree in all individuals. Some of these, however, gradually undergo more or less recovery in water and finally attain the condition of Figures 59 and 60. These, too, raise the question of possible re-ectodermization of the apical region. After 18–19 hours in hypertonic m/20 the condition ranges from that of Figure 58 to forms like Figures 61 and 62 and to some with complete obliteration of the blastocoel. With this continued exposure to the hypertonic solution there is in general less, and often no, actual dissociation into free cells, probably because of the high salt content; but epithelial pattern may completely disappear. Returned to water at this time, individuals stick together in indefinite numbers on contact and give rise to large masses—at first irregular in form, but gradually becoming or approaching spherical, bounded by an epithelium and filled with cell debris or cells, some of which often develop pigment or epithelial vesicles. Single individuals become spherical and anaxiate like Figure 56, some with others without epithelial vesicles in the blastocoel; they differ from the large multiple masses only in size. Axiate pattern appears to be completely obliterated in these cases.

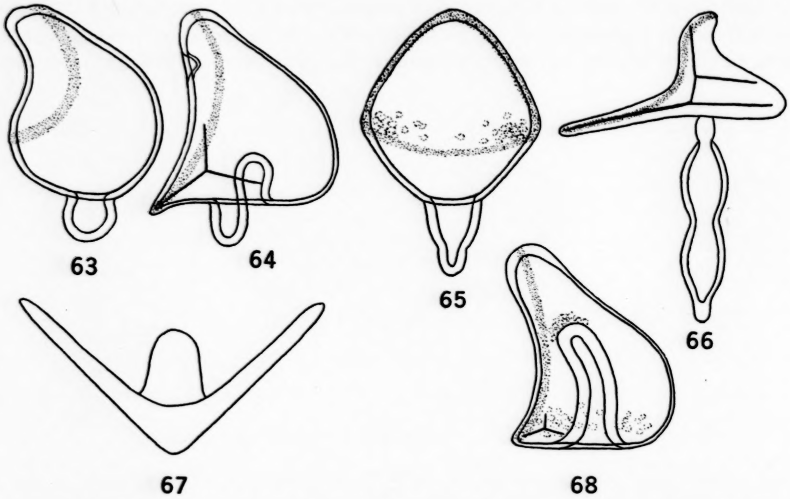


FIGS. 57–62.—Exposure from first cleavage to m/20 in sea water (hypertonic). Figs. 57 and 58, 7 hours in m/20, 12 hours in water; Figs. 59 and 60, 7 hours in m/20, 37 hours in water; Figs. 61, 62, 19 hours in m/20.

LITHIUM MODIFICATIONS WITH DIFFERENT EXPOSURE PERIODS BEGINNING AT LATER STAGES

It has long been known that, with a given concentration, exposures beginning at the earliest developmental stages are most effective in producing exogastrulation. A few data concerning effects of late exposures are given here in order to direct attention to certain facts.

With continuous exposure to m/60 beginning at first cleavage hypertonic sea-water solutions give 60–70 per cent entexogastrulae or exogastrulae with more or less entodermization of ectoderm. Similar exposure to approximately isotonic solutions of the same concentration entodermizes slightly or not appreciably; more or less invagination of the original entoderm occurs in all; and only some 10–20 per cent are entexogastrulae. Incidentally, this difference suggests that hypertonicity increases entodermization. With exposure to the same isotonic concentration from the beginning of mesenchyme immigration no entodermization and no exogastrulae, or in one series, 1–2 per cent, result. In isotonic m/50 continuously from first cleavage, more or less entodermization up to the basal half of the prospective ectoderm and exogastrulation occur in 85–100 per cent; but half or more of these are entexogastrulae with further development of the invaginated entoderm inhibited. With continuous exposure to the same concentration from the 6-hour blastula there is no appreciable entodermization, 98–99 per cent invaginate and develop into somewhat differentially inhibited plutei. The remainder are exogas-



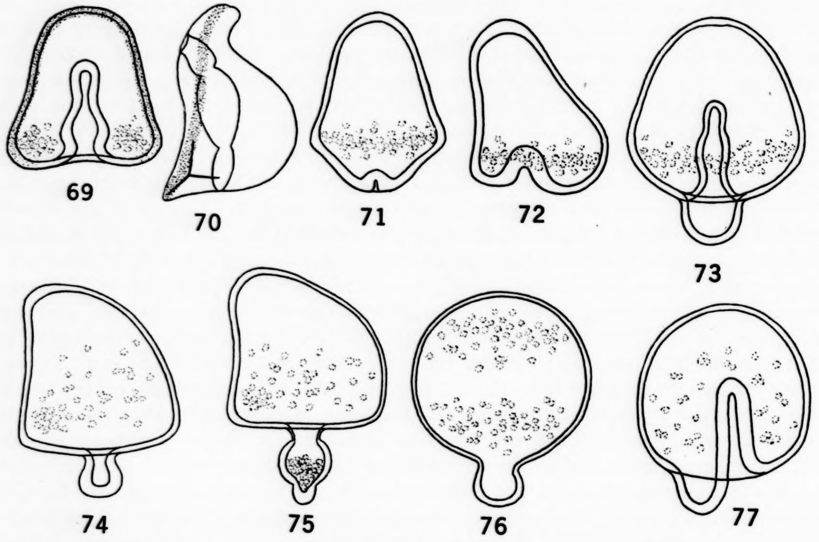
FIGS. 63–68.—Exposure from 5-hour blastula (Figs. 63–67) and from stage immediately preceding gastrulation (Fig. 68) to m/45 and m/40. Fig. 63, 14 hours in m/45, 24 hours in water; Figs. 64 and 65, 40 hours in m/45, 24 hours in water; Fig. 66, exogastrula, 14 hours in m/40, 25 hours in water; Fig. 67, outline from anal side of ectoderm of an exogastrula of the same lot as Fig. 66, indicating differential recovery; Fig. 68, condition after 11 hours in m/40 from stage immediately preceding gastrulation; entoderm more inhibited than ectoderm.

trulae but with small entoderm like those shown in following figures. Results are much the same with exposures of 14–40 hours to isotonic m/45 from the 5- to 6-hour blastula except that frequency of exogastrulae with small entoderm becomes higher. In these (Figs. 63–65) the entoderm remains small, shows little or no regional differentiation, while ectoderm develops ciliated band and sometimes stomodeal invagination (Fig. 64); and some individuals show secondary elongation of the apical region, indicating differential recovery (Fig. 63).

With 18 hours from first cleavage in isotonic m/40 and decrease in concentration to m/160 at that time, more or less entodermization results, and 100 per cent exogastrulae develop with large entoderm and with ectoderm ranging from differentially inhibited pluteus forms to completely radial in about 50 per cent. With 14 hours' exposure, beginning at the 5- to 6-hour blastula, entogastrulation and inhibited pluteus forms with more or less secondary modification by differential recovery are characteristic with a small percentage of exogastrulae of the type shown in Figure 66. The dorsally bent oral lobe is a very common feature of differential recovery following the less extreme inhibitions. The large evaginated entoderm, consisting wholly or almost wholly of the original prospective entoderm, also indicates differential recovery; at the end of the exposure period it was inhibited. Figure 67 shows the ectodermal outline viewed from the anal side in an exogastrula of the same type as Figure 66. Comparison of these results with those for m/45 above shows a greater degree of inhibition and less recovery for the same exposure period beginning at the same stage with the lower concentration. This is actually possible and has been observed not infrequently. During exposure the higher concentration inhibits development more than the lower, so that with the same exposure periods the return to water from the higher concentration occurs at an earlier stage of development than from the lower, and with the lesser degrees of inhibition the secondary modifications of differential recovery from the higher concentration may be greater than from the lower. Exposure to m/40 at beginning invagination does not produce exogastrulation in any case observed but does inhibit further development of entoderm to a greater extent than that of ectoderm. Secondary thickening and elongation of the apical region and development of the ciliated band take place while the entoderm remains without any regional differentiation (Fig. 68). Similar cases were described in an earlier paper (Child, 1936*b*).

As noted above, exposure to m/30 or m/25 for 5 hours or more from first cleavage is highly effective in producing exogastrulation with extensive entodermization, and with longer periods there is more or less dissociation of the original entoderm. The characteristic condition after 23 hours in m/30 from beginning gastrulation is shown in Figure 69. There is no entodermization and no exogastrulation; the invaginated entoderm is inhibited in development, particularly its apical region; but the ectoderm has developed a flat ventral side, and the ciliated band is distinguishable except ventrally. After return to water at this time, development is very slow; but 2 days later these forms have become small inhibited plutei with flat or somewhat convex ventral side, dorsally bent oral lobe, much inhibited, and short anal arms developing at wide angles up to 180° (Fig. 70). Slight differential recovery has occurred apicoventrally in the ectoderm and in the apical entoderm. Except for additional skeletal rods or spicules in some individuals, further development has not been observed.

Nine hours' exposure to m/25 from a stage immediately preceding gastrulation stops invagination in early stages (Figs. 71 and 72) or in the most advanced, with entoderm halfway to the apical pole. With continued exposure, gradual dissociation and death result without further development. With decrease of concentration to m/100 after 9 hours' exposure 40–50 per cent become entexogastrulae or exogastrulae with small entoderm, usually with more or less ectodermal ventrodorsality, but some with completely spherical ectoderm (Figs. 73–76). Some entodermal dissociation is evident in Figure 75, and the cells in the basal region of the blastocoel in Figure 76 are probably dissociated from entoderm, but in the lower concentration the entoderm has recovered to some extent and ceased to dissociate. The ventral side remains almost flat; no oral lobe, no arms (or only the earliest stages), and no stomodeum develop; and the entoderm remains blind and undifferentiated apically.



FIGS. 69–77.—Exposure to m/30 and m/25 immediately preceding or at beginning of gastrulation. Fig. 69, 23 hours in m/30 from beginning gastrulation, with ciliated band incomplete basally; Fig. 70, 23 hours in m/30 from early gastrula; Figs. 71 and 72, inhibition of invagination with 9 hours' exposure to m/25 from stage immediately preceding gastrulation; Figs. 73–76, 9 hours in m/25 from stage immediately preceding gastrulation, then in m/100; Fig. 77, entexogastrulation in recovery after 24 hours in m/25 from beginning gastrulation.

On return to water after 23 hours in m/25 from beginning gastrulation more or less recovery may occur. Some 20–30 per cent swimming at the surface may become small, differentially inhibited plutei. At the bottom 50–60 per cent remain differentially inhibited gastrulae, and 20 per cent become entexogastrulae or exogastrulae like Figures 73–77. In some of these with ventrodorsal difference in form the external entoderm is ventral to the invaginated, but whether this is always the case is uncertain in absence of visible ventrodorsality (Fig. 77).

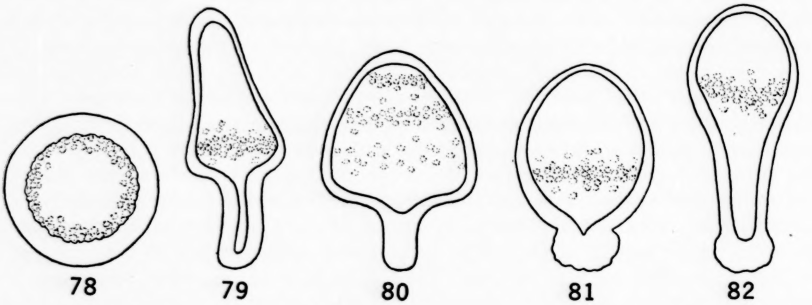
VARIATIONS IN SUSCEPTIBILITY IN *Dendraster*

It is well known that even in a lot of eggs from a single female, fertilized by sperm from a single male, developmental modifications by lithium and by other agents always differ more or less in degree in different individuals. With certain procedures 100 per cent exogastrulae result, but these differ in degree of modification. With less extreme effects the resulting forms may range from plutei of normal or almost normal proportions through various degrees of differential inhibition, with perhaps more or less secondary modification, to a variable percentage of exogastrulae, or even inhibited gastrulae or blastulae. Also, with different lots of eggs or embryos the same lithium concentration and exposure period at the same developmental stage usually produce a certain range of modifications, and the same is true for other agents. By no means all these differences, however, result from inherent individual differences in susceptibility to lithium or other agents. Aggregations on the bottom of the container result in different environmental conditions from those to which more completely isolated individuals are subjected, and in the aggregation conditions as regards oxygen and CO_2 and perhaps other products of metabolism may differ from the center to the periphery. Animals from such aggregations often show more extreme modifications than those more completely isolated. Aggregations of blastulae and gastrulae at the surface of the solution probably also have some effect on degree of modification. It is of interest in this connection that in aggregations of animals stained with methylene blue or Janus green reduction of dye often occurs without experimental decrease of oxygen. Even a few individuals close together may reduce more rapidly in regions in contact with others or toward the center of the group. On the other hand, if aggregations occur early in the exposure period before the agent has attained its full effect, dilution of the agent about the aggregation may result; and, if lack of oxygen or other incidental factors do not become effective, the aggregated individuals may be less modified than others. Here, again, results with dyes are of interest; aggregations become less deeply stained than isolated individuals in dye solutions. In short, without continuous agitation a rather wide range of environmental conditions is likely to arise in a container with developing animals. That such differences in environment may be important factors in determining different degrees of modification in the same container is indicated by the effects of crowding considered below. In general, the smaller the number of animals in a given volume of water or solution, the less extreme are these differences likely to be; but even under these conditions, environment is not necessarily uniform. That inherent differences in susceptibility are absent is not maintained, though the uniformity in rate of development in lots under good environmental conditions suggests that they are not ordinarily very great; and it is sufficiently evident that relatively slight differences in environment acting at a certain developmental stage may bring about considerable differences in course of development.

ENTODERMIZATION BY OTHER AGENTS

A considerable range of crowding of a large number of individuals in a small volume of water during early stages is highly effective in producing differential inhibitions of development which do not differ in any essential respect from the inhibitions produced by lithium. Differential inhibition of ectoderm, increasing inhibition of entoderm with approach of gastrulation, dissociation of the original entoderm, entodermization of prospective ectoderm, entexogastrulae, and exogastrulae are characteristic effects of crowding. Here only a few data are presented; extended comparative study of effects of different degrees of crowding has not yet been made.

With 16 hours' crowding from first cleavage the less affected are somewhat inhibited blastulae with the thick-walled basal region thicker and more extensive than in controls, suggesting entodermization. From these the forms range from early exogastrulae through blastulae with greatly thickened and extensive basal region from which cells are dissociating into the blastocoel, to spherical uniformly thick-walled blastulae with cells dissociating from the whole inner surface (Fig. 78), apparently completely anaxiate, and 10–20 per cent dead. A part of this lot taken from a layer at the bottom of the crowded container was returned to water after 16 hours' crowding. After 6½ hours in water 75 per cent of the living show more or less entodermization and develop as exogastrulae, usually with some degree of differential recovery of the apical ectodermal region and of the entoderm (Fig. 79). They finally become exogastrulae with ectoderm approaching more or less normal pluteus form and with long external entoderm, usually with three regions more or less differentiated. Individuals not infrequently show two distinct circles of cells, supposedly mesenchyme, at different levels in the blastocoel; and in a few animals indications of a third circle have been observed (Fig. 80). Since this condition has been seen, not during the period of crowding but during that of recovery, it suggests the possibility that the cell circles may represent progress in re-ectodermization of prospective ectoderm entodermized during the period of crowding. In most individuals an excess of cells is given off from a more extensive basal region than that of mesenchyme immigration under natural conditions. Those reaching a level near the apical pole may be localized at one stage; those farther basally, at another stage later. However, further investigation of these forms is desirable. The blastulae swimming at the surface in the same crowded lot, continued under crowded conditions up to 22 hours from first cleavage, gave only about 10 per cent exogastrulae, the remainder developing as plutei more or less modified by differential inhibition and recovery and similar to lithium forms.



FIGS. 78–82.—Developmental modifications resulting from crowding and recovery. Fig. 78, apparently anaxiate form with dissociation from entire inner surface after 16 hours from first cleavage in crowded culture; Fig. 79, exogastrula with some entodermization after 16 hours' crowding from first cleavage and 6½ hours in water; Fig. 80, exogastrula, 16 hours' crowding from first cleavage, 6½ hours in water, with mesenchyme localized at three levels. Fig. 81, early exogastrula with entodermization, 10 hours' crowding from early blastula; Fig. 82, exogastrula with entodermization, 10 hours' crowding from early blastula, 5½ hours in water.

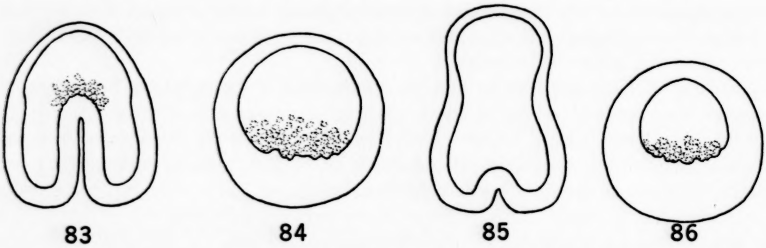
In another experiment after 10 hours' crowding from 6-hour blastula the level of mesenchyme localization indicated more or less entodermization in almost all individuals; and 50 per cent were already exogastrulae, usually with the original prospective entoderm a solid mass (Fig. 81). After 5½ hours in well-aerated water, 90–95 per cent were exogastrulae with the original entoderm, or most of it, forming a solid cell mass at the tip of the entodermized ectoderm in most of them (Fig. 82). Later ventrodorsality and finally inhibited pluteus forms with more or less apicoventral recovery developed from the remaining ectoderm, and the external entoderm increased in length and attained some regional differentiation. As far as could be determined, *Dendraster* exogastrulae resulting from crowding do not differ essentially in any way from those produced by lithium. The differential inhibitions are the same, and the secondary modifications of recovery show the same differentials.

Exposure of *Patiria* embryos to low concentrations of Janus green, beginning at first cleavage or even with blastulae at the beginning of movement, retards greatly or completely inhibits invagination, brings about dissociation of cells from entoderm, and entodermizes prospective ectoderm. However, with concentrations sufficient to produce these effects, most of the dye in the cells remains there, even after repeated washings; and since it is highly toxic, there is little or no recovery after return to water, and development does not proceed far beyond the stage of return.

With long-time exposures to very low concentrations of this dye the concentration of dye in solution decreases as it is taken up by the animals. In lots consisting of approximately standard numbers of animals in standard solution volumes of concentrations of 1/2,000,000 or less, all—or practically all—visible dye is removed from the solution by the animals within 30–40 hours; consequently, maximum accumulation in the cells must be approached or attained at that time or earlier.

Patiria embryos in Janus green, approximately 1/2,000,000 in initial concentration from first cleavage show, after 42 hours, great retardation of development in the living, and some 50 per cent are dead. In the least inhibited, ectoderm has not attained normal dimensions, entoderm is less inhibited than ectoderm and may invaginate and develop to the stage of apical enlargement, but dissociation of cells from its apical region is much more extensive than in normal formation of mesenchyme. In low oxygen, dye reduction occurs more rapidly in the invaginated entoderm than in the ectoderm; but the gradient in rate of reduction decreases acropetally instead of basipetally, as in controls (Child, 1936*a*), suggesting a differential inhibition, greatest in the apical entoderm and decreasing basipetally, as might be expected. The more affected individuals are smaller, invagination has stopped in early stages or has not begun, and there is more or less entodermization of prospective ectoderm and dissociation of cells from the original entoderm (Figs. 83 and 84). With this experimental procedure development stopped at these stages.

In another series in Janus green 1/2,000,000 for 30 hours from the blastula stage beginning movement, 40–50 per cent were essentially similar to Figure 85. In many of these cytolysis of the apical region was beginning. Others show somewhat more invagination and less entodermization. Death occurs with little or no further development. After repeated washings and 36 hours in water following exposure of early cleavage stages for 10 minutes to Janus green 1/50,000, invagination of entoderm does not take place; but the apicobasal differential in thickness of the blastula wall is greater than in controls, an extensive basal region becoming very thick, often with dissociation of cells into the blastocoel. The more extreme modifications are like Figures 84 and 86. There is no further development. Dissociation of cells from the basal region is very generally characteristic of inhibiting conditions in asteroids as well as in echinoids. Also, the extension of the thick-walled region acropetally suggests entodermization. If recovery and further development were possible, many of these forms would undoubtedly become exogastrulae. With the higher concentrations of LiCl earlier stages of *Patiria* and *Dendraster* exogastrulae are similar to these forms, except that the apicobasal differential in effect is often greater with lithium (see Figs. 53, 54, 57, 58, 61, and 62).



FIGS. 83–86.—*Patiria:* developmental modification by Janus green. Figs. 83 and 84, inhibited invagination of entoderm, dissociation of cells, and entodermization of prospective ectoderm after 42 hours from first cleavage in Janus green, initial concentration approximately 1/2,000,000; Fig. 85, entexogastrula with inhibited invagination of original entoderm and entodermization of ectoderm after 30 hours in Janus green, initial concentration approximately 1/2,000,000, from blastula stage beginning movement; Fig. 86, 10 minutes in Janus green, approximately 1/50,000 at early blastula, 36 hours in water.

DISCUSSION

Lithium chloride is highly effective in bringing about differential modification of echinoderm development, apparently because very considerable regional difference in degree of its inhibiting action may occur without death of any part. In other words, the regional difference along an axis in susceptibility to LiCl appears to be greater than to various other agents.

Inhibitory ectodermal modifications.—With exposure to effective ranges of lithium concentration, beginning shortly after fertilization, differential inhibition decreases from the apical or animal pole basipetally. The apical region remains relatively smaller, and its wall often thicker, than under natural conditions, while the basal region attains more nearly normal size in blastula stages. Comparison of Figure 1, normal, with Figures 15, 22, 28, and 29 shows this differential effect. With adequate concentrations and exposure periods ectodermal differential inhibition becomes apicoventral as development progresses. All degrees of inhibition of the oral lobe developing from the apical region and of the ventral side appear, resulting in modifications with oral lobe reduced or absent, and with a flat, or even convex, ventral side (Figs. 13, 14, 32, 33, and 46). As regards inhibition of the ventral side, different modifications result from different degrees of inhibition. Decrease of arm angle (Fig. 40), parallel or more or less fused arms (Fig. 21), or one-armed forms (Figs. 25, 26, 34, 37–39) may result. When exposure is continued or the inhibiting effect persists to later postgastrular stages, origin of local organ gradients of oral lobe and arms is more or less completely inhibited. With the more extreme differential inhibitions ventrodorsality may be completely obliterated, the ectoderm remaining completely radial, even after return to water.[4] With obliteration of ventrodorsality the mesenchyme does not become bilaterally localized. These differential inhibitions of ventrodorsality are entirely similar in principle to the modifications by differential inhibition with various agents of the planarian head,[5] the heads of fish embryos,[6] and amphibia (Bellamy, 1919). In those forms with increasing inhibition bilaterally localized organs of the cephalic region develop progressively nearer the median plane, become single in the median plane, or are entirely absent. The single apical coelom (Fig. 46) represents a similar mediolateral differential inhibition. With extreme differential inhibition axiate pattern may be completely obliterated from the ectoderm as far as any differentiation is concerned (Figs. 49, 51, 52, and 60). In these cases, however, the remaining ectoderm is only the apical part of the original prospective ectoderm.

Inhibitory modifications of prospective entoderm.—Although prospective entoderm is less inhibited than apical regions in earlier pregastrular stages, it becomes more susceptible as gastrulation approaches. If it invaginates, its further development is more or less inhibited, its apical region most of all. Coelom development may not occur, or a single apical coelom may develop without any evidence of bilaterality (Fig. 46). This is evidently another case of mediolateral differential inhibition similar to those of the anal arms, the planarian, the fish, and the amphibian head. Dissociation of cells from its more apical levels and extending basipetally may take place (Figs. 14, 16, 17, 32), or its apical portion may become a solid cell mass (Fig. 45), while ectodermal development proceeds further. With more extreme lithium action dissociation begins without invagination or with evagination. Parts of the prospective entoderm remaining undissociated appear to lose in part or wholly their epithelial arrangement and become a solid mass; or most or all of the entoderm may dissociate internally, externally, or both.[7] The solid cell masses at the entodermal tips of many exogastrulae are evidently parts of the original prospective entoderm which have not yet dissociated (Figs. 18, 41, 44, 48, and 49). The thick-walled tips of the entoderm of many exogastrulae may also be parts of the original entoderm with epithelial order retained or regained (Figs. 37, 39, and 47). In very high concentrations the loss of epithelial order progresses acropetally until the whole individual becomes a solid mass (Figs. 58, 61, and 62).

With a certain range of concentrations and with exposure periods beginning in later pregastrular stages or immediately before gastrulation, the original entoderm may invaginate or evaginate partially or wholly but is more inhibited in development than ectoderm (Figs. 63–65, 68, 69, 71–77). The evidence indicates that with approach of gastrulation the prospective entoderm undergoes a change in physiological condition rendering it more susceptible to lithium action than in earlier stages and more susceptible than ectoderm. This evidence agrees with that from differential dye reduction in low oxygen in indicating that prospective entoderm, as well as mesenchyme, undergoes a considerable activation preceding gastrulation. If this is the case, increase in susceptibility to lithium is to be expected.

[4] See Figs. 18, 24, 27, 30, 31, 35, 36, 41, 43, 44, 47, 48, 49, 51, 52, 59, and 60.

[5] Child, 1921, and earlier papers; Child and Watanabe, 1935.

[6] Stockard, 1907*a*, 1907*b*, 1909, 1910, 1914, 1921.

[7] See Figs. 15, 22–24, 28–31, 33–37, 39, 41, 42–44, 47–60.

Entodermization of prospective ectoderm.—That exposure to lithium in early stages may transform more or less, or in extreme cases all, of the prospective ectoderm into entoderm which constitutes a larger or smaller part of the external entoderm of exogastrulae appears evident. The question how this entodermization is brought about and whether it represents a specific lithium effect is of considerable interest. As already pointed out, Runnström and his co-workers regard lithium as enhancing and extending acropetally the effectiveness of a vegetal gradient, presumably by favoring or determining formation of the specific vegetal substance or substances postulated as constituting this gradient. With, or resulting from, this action there is a corresponding decrease in extent or at least in effectiveness of the opposed animal gradient. Actually, however, lithium appears to be a general inhibitor of development. It inhibits development of prospective entoderm, though in early stages less than that of prospective ectoderm, while at the same time it may entodermize prospective ectoderm. Moreover, as gastrulation approaches, prospective entoderm becomes more susceptible to, and so more inhibited by, lithium than ectoderm; but little or no entodermization occurs with exposure to lithium beginning in these more advanced stages. In short, lithium inhibits the original prospective entoderm, and entodermizes prospective ectoderm. It also inhibits more or less completely further development of entodermized ectoderm. In Figures 13, 14, and 16–18 there is considerable entodermization, but these figures represent the most advanced stages attained with continuous exposure to concentration of m/70 to m/50. Only after return from these concentrations to water at a stage near that of Figure 15 does entodermal development proceed farther. In higher concentrations inhibition of entodermized ectoderm, as well as of the original entoderm, is even more evident (Figs. 28, 29, 53, 54, 57, 58, 61, and 62). Development ceases early unless return to water or to a much lower lithium concentration occurs before the inhibiting action becomes irreversible.

The later in pregastrular development exposure to lithium begins, the less the degree of entodermization. With exposure beginning just preceding, or at the beginning of, gastrulation, there is little or no appreciable entodermization (Figs. 63–77), presumably because differentiation of ectoderm has attained a stage which makes it impossible under the conditions of the experiments; but further development is still more or less inhibited, and entoderm is more inhibited than ectoderm.

So far, then, it appears that lithium is generally inhibitory in its action on *Dendraster* development. If this is the case, the question at once arises whether its action in entodermizing prospective ectoderm may not also be inhibitory. Lithium is not the only agent that entodermizes prospective ectoderm. Crowding in early stages also entodermizes and produces exogastrulation and loss of epithelial order in the original entoderm (Figs. 79–82). It also decreases and obliterates ventrodorsality and even polarity in the ectoderm and, with more extreme action, brings about dissociation of prospective entoderm exactly as does lithium. It may even bring about dissociation from all levels of blastulae (Fig. 78). This also results with high lithium concentrations. At present there is no evidence to indicate that crowding has any other than an inhibitory action, but its differential effects are similar to those produced by lithium. The incidental data on *Patiria* show that Janus green is a highly effective inhibitor of development. In early stages its inhibiting action decreases basipetally; but it inhibits invagination more or less completely, dissociates entoderm, entodermizes prospective ectoderm, and also gives rise to exogastrulae. Except that it is much more toxic than lithium and, in concentrations within the cells sufficient to produce these modifications, stops development and kills in relatively early stages, its differential action on development is essentially similar to that of lithium. Here, again, there is no evidence to indicate anything but inhibitory action.

In view of these facts, it seems that we must assume either that crowding and Janus green have a specific effect on the vegetal gradient like that of lithium or that lithium, like crowding and Janus green, inhibits development generally but differentially and without regional specific action. In the light of all the evidence the second alternative appears the more probable.

If prospective entoderm originates from the lower levels of a primary apicobasal gradient, as dye reduction and differential lethal susceptibility indicate,[8] entodermization may result from depression or inhibition of prospective ectoderm below a certain "physiological level"; that is, the specific difference between ectoderm and entoderm may be a secondary result of a nonspecific, primarily quantitative difference. According to this suggestion, entodermization occurs first in the most basal levels of prospective ectoderm and progresses acropetally with increasing inhibition because lower levels of ectoderm require only relatively slight inhibition, higher levels, more extreme inhibition, to bring them down to the entodermal level.

The question has already been raised whether the forms with external entoderm originating from entodermized ectoderm are, strictly speaking, exogastrulae. Under natural conditions the entodermized ectoderm would have developed as ectoderm and would never have invaginated. Entodermization apparently has no relation to gastrulation, for entodermized ectoderm shows no tendency to invaginate, even after return to water, though the original prospective entoderm very often invaginates after conditioning or recovery (Figs. 13, 14, 17, and 25). Actually, these forms are differential modifications of prospective ectoderm, more or less entodermized; the original prospective entoderm may or may not take part in their formation. We might assume that, when cells of prospective ectoderm become entoderm, they acquire a polarity which, under natural conditions, would result in invagination and that lithium and other entodermizing agents reverse or obliterate this polarity. If that were the case, we should expect that entodermized ectoderm would invaginate after return to water at a certain time; but such invagination has not been observed. It may even be questioned whether the entoderm of exogastrulae derived from entodermized ectoderm is inside out. It remains in essentially the same relation to other parts as when it was prospective ectoderm, and there is no indication that the polarity of its cells has been reversed. The only reason for believing that it is inside out is the fact that it is external instead of internal. However, in view of established usage and for convenience, forms with entodermized ectoderm may be called "exogastrulae" in a purely descriptive sense, because their entoderms are external; but it should not be forgotten that, as regards origin, they differ widely from true exogastrulae, in which the original entoderm evaginates instead of invaginates.

Mesenchyme.—Except in very high concentrations (Figs. 58, 61, and 62) mesenchyme immigrates; but only in slightly inhibiting concentrations does it become bilaterally localized during exposure, though this localization may follow return to water or may appear secondarily in conditioning. In individuals with entodermization mesenchyme is mostly localized in a circle at, or slightly apical to, the boundary between remaining ectoderm and entodermized ectoderm (Figs. 15, 22, 28–31, 42, 50, and 55). Cells dissociated into the blastocoel from entoderm resemble mesenchyme, but whether they play any part in further development is uncertain. Perhaps some of them may develop as mesenchyme; but most, if not all of them, usually appear not to develop further except when they form entodermal vesicles in recovery (Figs. 51, 55, and 56). With obliteration of ventrodorsality mesenchyme remains in a circle; and, if skeleton develops, it is merely an irregular circle of spicules (Figs. 35 and 41). With further reduction of ectoderm mesenchyme is merely aggregated without order in the ectodermal blastocoel and sometimes with recovery develops a single rod, often longer than the ectoderm (Figs. 48, 49, 51, 52, 59, and 60). With complete inhibition of skeletal development abundant pigment may still develop. The small ectodermal regions of the more extreme exogastrulae (Figs. 48, 51, 52, and 60) usually become heavily pigmented (not shown in figures). Normal skeletal pattern develops only in very low concentrations or in recovery after short exposure. In general, with increasing inhibition skeletal development becomes increasingly aberrant and incomplete or absent. Evidently orienting factors in the ectoderm become less effective or are obliterated, and the mesenchyme cells become less able to form skeleton, though they may regain the ability to some extent on recovery.

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[8] Child, 1936*a*, 1936*b;* see also Child, 1915, 1916*a*, 1916*b*.

Secondary modifications.—With continuous exposure from early stages to a certain range of low concentrations development is primarily retarded with slight differential inhibition; but a differential tolerance, acclimation, or conditioning may gradually become evident in secondary differential modifications opposite in direction to the primary differential inhibitions. After return to water following exposure, even to strongly inhibiting concentrations, modifications similar in character but often greater in degree may result. After slight inhibitions the secondary modifications may be wide-angled plutei with large oral lobes and small body, either in differential conditioning (Fig. 6) or in differential recovery. These are, in general, similar to secondary modifications in *Arbacia* obtained with other agents (Child, 1916*b*). With inhibition sufficient to produce exogastrulae differential tolerance, conditioning (Figs. 13 and 14) or recovery (Figs. 32, 33, 46, and 67) may result in more or less ectodermal modification.

Secondary modifications also occur in the entoderms of exogastrulae. For example, in m/35 from first cleavage development does not proceed far beyond stages shown in Figures 22 and 23; but after return to water, forms like Figures 24–27 develop. Figures 28–31 show development with continuous exposure to m/30; Figures 32–36, forms developing in water after various exposures to m/30. Figures 45–49 show differential recovery from m/25, a still more inhibiting concentration with continuous exposure. The very large entoderms of the more extreme exogastrulae (e.g., Figs. 33–37, 39, 41, 47–49, 59, and 60) are not direct effects of lithium but are results of differential recovery from lithium inhibition. Some relative increase in size of entoderm may appear in differential tolerance or conditioning (Fig. 18), but development of exogastrular entoderm is greatest in recovery, and most or all of this entoderm may be entodermized ectoderm. Apparently entodermization may be followed after return to water by more or less activation of the entodermized region: this is also indicated by dye reduction (Child, 1936*b*).

Entoderm which has become a solid cell mass may apparently regain epithelial order to some extent with recovery. In water after inhibition by high concentrations, epithelial vesicles often appear in the blastocoel (Figs. 51, 55, and 56), though not present during exposure. Also, the solid masses at the entodermal tips of exogastrulae not infrequently appear gradually to become epithelial with continued life in water. Development in recovery of forms like Figures 59 and 60 from individuals like Figures 57 and 58 shows how great the secondary modification of recovery may be. With continued exposure to lithium death results without development beyond the stage of Figures 57 and 58. However, in these particular cases development of single individuals has not been followed through.

There are many indications that prospective ectoderm after entodermization or approach to entodermal condition may finally develop as ectoderm in secondary modification. In many exogastrulae with more or less skeletal development after return to water, the skeleton, instead of being localized about the anal side of the ectoderm, forms nearer the apical pole (Figs. 19, 35, 37, and 41). Also, both in differential tolerance or in conditioning in low concentrations, and in differential recovery, arms often develop at levels farther apical in the ectoderm than their normal position and the ectodermal region basal to the arm-level is enlarged and convex (Figs. 13, 14, and 33). In many exogastrulae without skeletal development the circle of mesenchyme is localized much farther apical than its usual position, even near the apical pole (Figs. 36, 44, 47, and 55). Moreover, two distinct circles of mesenchyme appear in some individuals; and in a case from a crowded lot, indication of a third circle was observed (Fig. 80). These cases suggest a primary entodermization sufficient to determine localization of mesenchyme and, with secondary modification, a re-ectodermization of the more apical levels. However, if this is the case, the mesenchyme does not migrate basipetally but remains at the level determined by the primary entodermization. In those individuals with two or three circles at different levels part of the mesenchyme may have been localized at different stages of entodermization or re-ectodermization, perhaps with increase in cell number during recovery. If inhibition or depression, rather than formation of a specific substance or substances, is the primary factor in entodermization, it seems probable that, if the inhibiting factor is removed early enough, before definitive differentiation, the cells concerned may regain or approach their original condition. Since return or approach to the original condition certainly does take place in many cells with tolerance, conditioning, or recovery after primary inhibition by lithium and other agents, its occurrence here presents no difficulties. Re-ectodermization has been reported by von Ubisch (1925, 1929) in a sea urchin following lithium treatment with isolation of parts.

Mesenchyme may fail completely to develop skeleton in LiCl but may regain its ability to do so after return to water, but form and distribution of skeleton differs widely with degree of axiate pattern present in the ectoderm. With differential recovery from less extreme inhibitions excess of skeleton may develop with formation of three, four, and even five arms or an indefinite accumulation of spicules and rods in the body. The relative roles of ectoderm and skeleton in development of supernumerary arms have not been determined; but in the cases observed, the ciliated band and the arms develop in the usual relation.

In general, regions primarily most inhibited by lithium show secondarily the greatest development in differential conditioning and recovery unless they have been so greatly inhibited that secondary modification becomes impossible before development has attained its most advanced stage. The forms resulting depend on exposure period as well as on concentration. The local activations associated with development of oral lobe and anal arms take place normally at certain stages of development. With exposures beginning in early stages the apical region may be so inhibited that an oral lobe is completely absent, even with recovery; but more or less arm development may still be possible after return to water or with conditioning.

Reconstitution in exogastrulae.—In those exogastrulae with partial entodermization of ectoderm, development of the remaining ectoderm involves extensive reconstitution, except when axiate ectodermal pattern is completely obliterated, as in Figures 27, 43, 49, 51, 52, and 60. Scale of organization decreases with decrease in size of ectoderm, and everywhere except in the extreme apical region the various parts differentiate in cells which normally would differentiate otherwise. A modified pluteus body may differentiate from the apical half, or even less than half, of the original prospective ectoderm. In these cases the anal arms develop from regions which normally would give rise to parts intermediate between arms and oral lobe. Whether or to what extent localization and outgrowth of arms are determined by skeletal development or by ectoderm in these forms is not known. In normal development the ectoderm is concerned in localizing the mesenchyme, but in these partial ectoderms the arms develop from ectoderm of quite different prospective significance. Since ventrodorsality is still present and arms develop in definite relation to it in these cases, it appears probable that even these partial ectoderms play some part in localizing mesenchyme and skeleton. However, in some exogastrulae with a single longitudinal skeletal rod, as in Figures 52 and 59, a short armlike outgrowth with the skeletal rod extending to its tip may develop from the apical pole; but it may be questioned whether this is, strictly speaking, an arm or merely an outpushing of the ectoderm by elongation of the skeletal rod.

Although isolated apical halves of early stages do not gastrulate and develop complete individuals under natural conditions (Hörstadius, 1935), it is evident that less than an apical half of the ectoderm in exogastrulae may develop parts normally arising from regions farther basal and may approach more or less pluteus form. It was shown by von Ubisch (1925) that apical halves, after treatment with lithium, may even develop entoderm and gastrulate. If these ectodermal reconstitutions in exogastrulae and isolated apical halves were due to increase of a vegetal gradient of specific substance or of some other kind, we should expect to find more extensive reconstitution of basal (vegetal) regions by the remaining ectoderm of exogastrulae with increase of lithium effect, at least up to a certain limit; but actually such development decreases. Development of arms is inhibited, ventrodorsality and even polarity do not appear or are obliterated with increasing lithium action; but with recovery, even from exposure periods and concentrations which stop development in its earlier stages, more or less ectodermal reconstitution may take place.

In terms of the gradient concept advanced in this paper, reconstitution of more basal body-levels by partial prospective ectoderms of exogastrulae and isolated apical halves results from differential inhibition with shortening and lowering of physiological levels of the primary apicobasal gradient, with consequent decrease in scale of organization, so that the partial ectoderm approaches, or in the isolated half may attain, wholeness in development. This effect is not specific for lithium; crowding in *Dendraster* and other agents with other species give essentially the same results. The alteration of the primary gradient is a direct effect of lithium or other agents; but the reconstitution, with further development than is possible in lithium, and origin of an archenteric gradient in isolated, lithium-treated halves and of arm gradients in exogastrular partial ectoderms, usually, if not always, involves secondary modifications resulting from differential tolerance, conditioning, or recovery, as clearly shown above.

Hydroid reconstitution shows essentially similar conditions. A piece of *Tubularia* or *Corymorpha* stem so short that under natural conditions it gives rise only to a hydranth or to the apical region of a hydranth may, after exposure to inhibiting agents, develop a smaller complete hydranth and stem, and in *Corymorpha* a basal holdfast region also. The gradient determining reconstitution is, under natural conditions, on such a scale that only its more apical levels are present in the short piece, and only a hydranth or the more apical levels of a hydranth develop. Inhibiting agents lower its levels differentially and shorten it; consequently, the scale of organization is decreased, and hydranth and stem or a complete individual on a smaller scale develop from the piece (Child, 1931, and unpublished data). Conversely, increase in scale of organization may result from elevation of temperature. Since these alterations in scale of organization in hydroids are produced by various agents, there is no ground for believing that they result from specific effect of a particular agent on a particular substance or substance gradient, and it does not appear at present that such a hypothesis is necessary to account for decrease in scale of organization in echinoderm development.

The small thin-walled "neck" often present between what would have been the anal region in normal development and the external entoderm (e.g., Figs. 14, 19, 24, 25, 27, 39, 41, 43, and 44, etc.) also represents a reconstitution and probably always a secondary modification. It is apparently ectodermal in origin, and the entoderm often separates from it after return to water, leaving it attached to the remaining ectoderm. If entodermized ectoderm is not entoderm inside out, its attached end may represent its apical end; the higher rate of dye reduction at this end in most exogastrulae of this type (Child, 1936*b*), as well as its origin from ectoderm, in which levels nearer the apical pole represent higher gradient levels, adds support to this suggestion. In this connection it is perhaps a point of some significance that the usually flattened basal region of the ectoderm in partially entodermized forms is usually the region of highest rate of dye reduction in the ectoderm, except when secondary apical modification and outgrowth occur (Child, 1936*b*); that is, in these exogastrulae the relations of the basal ectoderm to other parts are apparently more or less like those of regions about the apical pole in normal development. Under these conditions development of a stomodeum from the prospective anal region does not appear improbable. Reconstitutional development of a ciliated band in circular form around the basal region of radial exogastrular ectoderms (Figs. 24, 35, 41, and 44) is probably also associated with this change in the ectodermal gradients. With recovery after obliteration of ventrodorsality the physiological condition of the basal ectoderm may become enough like that of the normal ventral region to permit development of the ciliated band about its margin.

This neck may develop, however, in forms with little or no entodermization. In these the attached end of the entoderm is presumably the anal end; and the neck might be regarded as a proctodeum, developing under the experimental conditions, although absent or not developing appreciably under natural conditions. But it does not seem necessary to assume that it should always be either proctodeum or stomodeum: the important point is that it is a development of ectoderm, a reconstitution, resembling a proctodeum or stomodeum.

In exogastrulae with entoderm consisting in part or wholly of entodermized ectoderm, entodermal reconstitution is also involved. Entodermization is itself a reconstitution; but there is often further reconstitution, particularly with secondary modification. If the original prospective entoderm is not completely dissociated, it may form only the tip of the exogastrular entoderm, instead of the whole; but development of the whole entoderm is orderly and definite, as far as it goes. With secondary modification, particularly with recovery, two or three entodermal regions, separated by constrictions, may develop (Figs. 19, 24, 33–35, 37, 39, 41, 47–49, 52, 59, and 60). This development takes place in part or wholly in tissue originally prospective ectoderm and evidently results from extensive and orderly reconstitution. The original entoderm, as far as it takes part, forms less than normally, and development of entodermized ectoderm differs completely from its normal fate. How such an orderly and harmonious development is determined in cells of very different prospective significance is an interesting question. If it were the result of increase in concentration and effective extent of a vegetal substance gradient, we should expect that the original prospective entoderm would develop more entoderm instead of less than normally. To account for this orderly and definite development, a definite pattern of relations between parts somewhat similar to that determining entodermal development in the normal animal seems necessary. In order that the original prospective entoderm, or some part of it, shall develop as merely a portion of exogastrular entoderm, its development must be controlled by factors in others parts. Development of regional pattern in exogastrular entoderm is more or less completely inhibited by lithium, except with differential tolerance or conditioning in low concentrations; only with recovery does it attain its fullest expression. With entodermization of the entire ectoderm, development of this pattern has never been seen in any individual of any species with which the writer has worked. Apparently the differences in physiological condition and the relations between parts which determine orderly axiate pattern may be completely obliterated in the original entoderm, in entodermized ectoderm, and in ectoderm by lithium and other inhibiting agents (Child, 1916*b*). If these relations originate in a physiological gradient or gradients, lithium and various other agents apparently obliterate the gradients by differential inhibition. If not completely obliterated, they may attain some degree of developmental expression with secondary modification. In exogastrulae the gradients and relations of parts associated with them are so altered that extensive reconstitution results.

THE PHYSIOLOGICAL-GRADIENT CONCEPT

The theory that axiate pattern in its simplest terms consists in a physiological gradient or gradient system in which differences in rate, rather than in kind, of metabolism are the primary active and effective factors in initiating and carrying on actual development does not assume that such gradient patterns are independent of a material substratum. It merely holds, first, that a gradient pattern may be initiated without pre-existing specific regional organization in the materials of the substrate in which it appears. The fact that physiological axes determining the reconstitution of individuals with a completely new organization can be established at practically any body-level in hydroids, planarians, and various other forms by a local activation or a purely quantitative external differential without definite relation to a pre-existing organization provides much evidence in support of this conclusion and none against it.

Second, the theory holds that differences in concentration of a multitude of substances must at once begin to appear at different levels of such a gradient in association with the different rates of reaction. Differences in rate of transformation of nutritive material and use of oxygen in relation to intake or presence in the protoplasm, in production of CO_2 and other metabolites, and in synthesis and breakdown of protoplasmic components provide almost infinite possibilities for the origin of specific material differences at different levels of such a gradient. With these reactions and their products are associated differences in water content, in ionization of various electrolytes, and in factors associated with the colloid substrate. Even if a physiological gradient is purely quantitative at the moment of its establishment, it seems impossible that it should remain so for any considerable length of time. The theory maintains that such a gradient provides an adequate basis for definite future differentiations at its different levels, both through the specific organization resulting directly from it and as a factor making possible realization of different gene potentialities in different regions or cells. If "determination" of a part in the course of development represents attainment of a certain degree of chemical or structural specificity in that part, the question at once arises: How did that part differ from others preceding its determination for a particular developmental fate? It must have differed in some way; otherwise it could not become determined as a certain part or organ. Can the primary differences between it and other parts have been anything but quantitative? Experimental investigation of amphibian development, for example, shows progressively less evidence of specificity in different regions as we pass from later to earlier stages. This does not necessarily mean that the amphibian egg at the beginning of embryonic development possesses nothing but a quantitative gradient pattern; but it may mean that, if we could extend experimentation back into the period of ovarian development of the oöcyte, we should come finally to a quantitative pattern. Determination of a part in development appears to be a gradual process and in general becomes progressively more stable: this certainly indicates a progressive increase in specificity.

Objection to the concept of a primarily quantitative axiate pattern with rate of basal metabolism as the active, effective factor has been raised on the ground that, if this were the case, differential modification should result from change in temperature. The answer to this objection is that with a sufficient change in temperature such modification does occur in various organisms. Low temperature inhibits differentially reconstitution of the planarian head and may produce all degrees of the graded series of forms—teratophthalmic, teratomorphic, anophthalmic, and acephalic—in pieces which at higher temperatures develop normal heads. In these cases the differential inhibition is most conspicuous mediolaterally, inhibition decreasing laterally from the median region; but it also decreases from anterior to posterior levels. High temperature increases not only rate of reconstitution and size of head but also frequency of normal heads on pieces which develop differentially inhibited heads at lower temperatures. In some individuals it also determines heads with second right and left eyespots, each lateral to the normal one, with corresponding greater development laterally of the cephalic ganglia. Moreover, it may bring about metamorphosis of teratomorphic heads with a single median eyespot and cephalic lobes approximated to, or in, the median plane, into heads with two eyespots in normal position right and left of the median plane in addition to the median eyespot, reduction of the cephalic lobes near or in the median plane anteriorly, and development of new lobes right and left in normal position; also, increased development of the cephalic ganglia. Animals conditioned to low temperature before section reconstitute normal heads in higher frequency when temperature is raised following section than those not so conditioned; and with conditioning to high temperature before section, the frequency of differentially inhibited heads is higher at low temperature than in pieces from animals not so conditioned. With recovery from the differential inhibition of low temperature, further development of differentially inhibited head-forms may occur with more or less approach to normal.[9] Differential modifications of echinoderm development have also been produced by change of temperature (unpublished).

However, the possibilities of developmental modification by temperature have not been extensively explored. Amphibian embryos have been subjected to temperature gradients, and effects of different temperatures on development of the fish and chick have been recorded; but really analytic investigation of temperature in this relation to embryonic development has not yet been undertaken. It is perhaps not necessary to point out that, after specific regional "determinations" have originated in an egg or embryo, differential modification by temperature may still be possible to some extent because quantitative gradient factors do not necessarily disappear with occurrence of determination; but we might expect the modifications to be less extreme than in stages preceding determination and different in character as new gradients appear. In *Dendraster* the most extreme lithium modifications are obtained with exposure at the earliest stages, but differential modifications are still possible with exposure beginning in gastrula stages when ectoderm and entoderm are apparently rather stably determined. To produce differential modification a gradient must apparently be differentially altered; the differentials of different levels must be either increased or decreased. Investigation of temperature coefficients of chemical reactions indicates that they do not remain constant at all temperatures, even within the physiological range; and temperatures above and below this range become differentially injurious or lethal. Certain difficulties are associated with use of low temperature for differential developmental modification. In planarian pieces, for example, conditioning to low temperature may occur, even though development is practically at a standstill; consequently, there may finally be less differential inhibition than expected. Also, low temperatures may approach or fall below the threshold of development; and temperatures that freeze the organism are, of course, useless for experiment of this sort. High temperatures beyond a certain limit are inhibitory or lethal, except as conditioning may take place. The chief difference between temperature and other physical and chemical agents as regards differential action on development seems to be the range within which differential modification does not appear or is slight. Two factors, and perhaps others, may be concerned in determining this range. First, within it the whole gradient system may be elevated or lowered without much alteration of the differentials of different levels. There is no reason for believing that equal change in rate of reaction in the whole system will modify development differentially, though it will accelerate or retard, and may increase, scale of organization. Second, changes in temperature are features of natural environment, and most organisms have attained a relatively wide range of tolerance and become rapidly conditioned to these changes or recover rapidly from them; consequently they produce little or no modification.

As a third point, the gradient theory holds that, even though specific regional differences are already present to some extent along a gradient, differences in rate of the basal metabolic reactions may still be important factors in determining form and proportion, and perhaps even course of differentiation. These differences in rate may be altered nonspecifically by external agents, whatever the particular way in which a certain agent brings about the alteration; and differential modification of form and proportion may result even though determination of parts is already advanced.

Fourth, the theory does not maintain that axiate pattern of eggs at the beginning of embryonic development is always the purely quantitative primary pattern. Obviously, it is not. The egg is a more or less highly specialized cell, and in many eggs a considerable regional differentiation is already present at the first cleavage. Even in some of those, however, quantitative gradient differences are still present and are essential factors in development, and extensive differential modification of development is still possible. The interpretation of exogastrulation in the present paper does not, by any means, exclude the possibility that the two opposed specific concentration gradients postulated by Runnström are present in the echinoderm egg. It is merely based on evidence indicating that, whether they are or are not present, lithium does not act specifically or exclusively on one of them but is rather a differential inhibitor of development in general, the degree of inhibition depending, in some unknown way, on difference in rate of vital activity rather than on concentrations of specific substances. Concentrations in themselves without metabolism can accomplish nothing in development; and two opposed concentration gradients, such as Runnström postulates, may be associated with, or result from, a single gradient in rate of metabolism; that is, a single gradient in rate of metabolism may coexist with decrease of one and increase of another substance gradient in a certain direction. The evidence from differential modification of development and differential dye reduction indicates a single gradient in rate, decreasing basipetally, in early stages of echinoderm development, with a secondary gradient in the opposite direction appearing in the basal region as gastrulation approaches, or in the starfish somewhat later, and local gradients of arms and oral lobe appearing still later. It does not, however, give any information as to presence or absence of the concentration gradients of Runnström.

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[9] Child, 1912, and unpublished data; Behre, 1918.

Fifth, the organization of the eggs of many organisms appears to be an incidental, rather than a fundamental, factor in development—a factor determined by the differentiation of the egg during its ovarian history, maturation, and fertilization. The ascidian egg, for example, evidently possesses a relatively high degree of orderly regional differentiation before the first cleavage begins; but an ascidian can develop from a bud at the tip of a stolon or elsewhere, from an isolated piece of ascidian body or stolon, and from the cell aggregates formed in many species within the bodies of degenerating individuals. There is no evidence of an organization similar to that of the egg in these various forms of development; but they may all give rise to ascidians like those from eggs. Evidently the fundamental factors of ascidian pattern originate in some way in all. Are those factors primarily anything more than gradients in rate of living? A bud is apparently a local activation decreasing from a center; there is no evidence at present that it is primarily anything more than that. In reconstitution from pieces of hydroids, planarians, and many annelids a new organization originates and determines more or less the making-over of the old. Buds and reconstitution of isolated pieces or of aggregates of dissociated cells bring us closer to the beginnings of developmental pattern than do eggs of most animals. We must look to these forms of development for the fundamental factors of developmental pattern. Only by investigation and comparison of all the different forms of development can we hope to distinguish the fundamental factors of pattern from those which are incidental to a particular form of development. Many eggs at the beginning of cleavage are actually well along in development; but in the bud and in reconstitution of a new whole individual from an isolated piece or a cell aggregate, development of pattern begins anew.

Some biologists apparently believe that metabolism is not a fundamental factor in development. For example, Shearer (1930, p. 266) says: "Morphological organization has nothing to do with metabolism"; Parker (1929, p. 424), in criticizing the physiological gradient concept, makes the statement: "The metabolic activity of the organism is not a true formative process, but the result of such a process." Spemann (1938, pp. 321 ff.) seems to hold somewhat the same opinion; his discussion of gradients involves the mistaken assumption that, according to the gradient theory, there must be only quantitative metabolic differences in the amphibian egg at the beginning of embryonic development. In his recent book Weiss (1939, pp. 373–83) seems to hold that specifically different capacities for organization exist in different regions independently of metabolism. Granting that these capacities are present in many eggs, how did they arise except through metabolism? And, even if they are present in eggs, are they present in the early stages of buds, of pieces of *Tubularia* or *Corymorpha* stem, or in aggregates of dissociated *Corymorpha* cells? In isolated pieces of the planarian body, of various annelids, and of some ascidians development of a particular part has no definite or constant relation to a particular region of the original individual or to a pre-existing organization; but a new organization originates. Does it originate independently of metabolism? If we inhibit metabolism, it does not appear; and in many cases it is possible to initiate its development by purely quantitative external differentials which in one way or another determine gradients involving metabolism. It has been pointed out repeatedly that, even if these gradients are primarily quantitative, they probably do not remain so for any great length of time, though quantitative factors may continue to exist and be effective, even in many adult animals. How do the regional organizing capacities and the presumably specifically different metabolisms of hydranth, stem, and holdfast region in a reconstituting piece of *Corymorpha* originate, if not through metabolism? Since any level of a new individual may develop from any level of the stem, these capacities are certainly not localized preceding isolation of a piece. In the absence of a gradient involving metabolism they fail to appear; with establishment of a sufficient gradient at any stem-level or at either end of a piece they do appear. The arguments of Spemann and Weiss are based on the egg and embryonic development rather than on development in general, but other forms of development throw more light on the origins of developmental pattern than does embryonic development at present. Like many other embryologists, they maintain that organization or specific capacity for it is present, but they do not tell us how it originates. If organization consists in localized presence of specific substances, how can these substances originate and be localized except in the final analysis through metabolism? Probably no one now believes that they are all present in the primitive germ cell. It is difficult to believe, for example, that localized formation of specific substances in the dorsal inductor region of the amphibian egg and embryo can take place independently of metabolism. Without metabolism ovarian development of the oöcyte and embryonic development cease. Even the differentiation of various organs and tissues does not persist if their metabolism is decreased below certain levels. How are electric and other physiological regional differences established and maintained except through metabolism? What is the possible nature of formative processes assumed to be independent of metabolism? In short, is there any more fundamental characteristic of living protoplasms than metabolism? At present, evidence of any such characteristic seems to be wholly lacking. It may be argued that structure of some sort is more fundamental, but structure without metabolism is not living protoplasm and accomplishes nothing that can be regarded as organismic development. Even if the organization of the egg originates in the orientation of elongate dipolar and symmetrical or asymmetrical protein molecules, as Harrison (1937) and others have suggested, the question at once arises: To what do the molecules orient? Metabolism is going on continuously in the ovarian oöcyte and in the parent organism to which it is attached. Can the orientation possibly occur without relation to this metabolism? In this connection, also, it is highly significant that in reconstitution of a piece of *Corymorpha* or of a planarian the new organization follows certain changes in metabolism in the region concerned and without them does not develop. Doubtless changes in structure are also involved and affect metabolism, but metabolism is the active and effective factor. There is, at present, no evidence indicating that the changes in metabolism characteristic of the new organization result from orientation of protein molecules in all the thousands of cells concerned. The polarities of the individual cells of the hydroid and planarian are apparently determined by local surface-interior differences without direct relation to the axiate pattern of the whole, but these cells become parts of the axiate pattern of the new individual and still maintain their original polarities. If their molecules are, or become, oriented, are they oriented with respect to the local surface-interior factors or to the axiate pattern of the whole individual? If they are locally oriented, then the new pattern is independent of them. If the new pattern is determined by their reorientation, how are their original polarities maintained?

Weiss, in his book, has much to say of the field concept, of field energy, of strong and weak fields, and of decrease in field energy from a center; but he does not tell us what a field is or may be as an active and effective factor in development, nor does he say what makes a field strong or weak or what the source and character of its energy may be. What is the basis for decrease of field energy from a center to periphery of the field? New fields originate in the course of development; how do they arise? Is their origin independent of metabolism? Is there some other source of energy in a field than metabolism? Without information or at least hypothesis concerning these points, the field concept remains almost mystical in character. It does provide a formal designation for particular regions of a developing organism in which particular results appear, or may appear, under experimental conditions. Much has been learned from transplantations and other lines of experiment concerning extent and times of appearance of certain fields in certain organisms, but little concerning their nature. Until the field has content of some kind which can accomplish the observed results, the field concept remains at best a purely descriptive and formal summary of observation and experiment, or, as the term "field" is often used, little more than a verbalism, a symbol of something unknown. It does not advance our knowledge of the physiology of development. Is a field in its dynamic aspects primarily anything more than a gradient system of a particular kind of metabolism in a particular kind of protoplasm? Can it, in the light of all the lines of evidence available, be anything more than that? Organ fields evidently originate in relation to a more general pattern; and, proceeding from later to earlier stages, we finally come to the field, pattern, or gradient system of the individual.

That organization with localized specific differences of substance and metabolism does originate in some way and that dynamic, not merely structural, factors are essential to its origin appears beyond question; that regional specificities originate and increase during development is indicated by many lines of evidence. If metabolism is merely incidental to, or a result of these changes, it would seem that we must again postulate a specific vital energy; Driesch's entelechy will scarcely serve our purpose, for that was conceived not as a source of energy but rather as controlling energy transformation—and that in living protoplasm is, in the final analysis, controlling metabolism.

The concept of physiological gradients in terms of dynamic factors effective in bringing about development, rather than of purely structural factors, does not in any way conflict with the concept of organization in terms of specific, regionally localized material differences. It merely maintains, on the basis of many lines of evidence, that such organization is not the primary pattern of development but the result of metabolic activity in a primarily quantitative pattern from which the regional specificities gradually developed. In short, this concept is an attempt to look beyond organization already present and to throw some light on the problem of its origin and development.

SUMMARY

1. Exposure to LiCl in the earliest stages of *Dendraster* development inhibits prospective ectoderm more than prospective entoderm. As gastrulation approaches, its inhibiting action on entoderm increases and becomes greater than on ectoderm. It may bring about partial or complete breakdown of entodermal epithelium into a solid cell mass or a dissociation into free cells.

2. All degrees of differential inhibition of ectodermal development result from lithium action: pluteus forms with arm angle decreased, more or less parallel or fused arms, single median arms, oral lobe partially or completely inhibited, complete inhibition of arm development, obliteration of ventrodorsality and even of polarity.

3. With sufficient concentration and exposure period lithium entodermizes prospective ectoderm progressively from basal levels acropetally, but it also inhibits further development of the entodermized ectoderm, as well as of the original prospective entoderm. Development of skeleton by mesenchyme is also decreased or completely inhibited.

4. Entodermization of ectoderm is apparently a result of inhibition or depression in the primary apicobasal gradient. It appears first in basal ectoderm because less inhibition is necessary to entodermize these than higher levels. In short, lithium is apparently a general and highly effective inhibitor of development. There is no evidence of specific regional effects of lithium on early development. Crowding produces similar differential inhibition and entodermization in *Dendraster;* and Janus green, in the starfish, *Patiria.*

5. The evidence indicates that exogastrulation results primarily from nonspecific differential inhibition rather than from specific regional effect of lithium or other agents, but secondary modifications of exogastrulae with great increase in size of entoderm may result from differential tolerance, conditioning, or recovery.

6. Two types of differential modification are commonly called exogastrulae. In true exogastrulae the original prospective entoderm evaginates instead of invaginating. In other, so-called exogastrulae the original entoderm may invaginate, break down into a solid cell mass, or undergo more or less complete dissociation into free cells. In these forms the external entoderm consists largely or wholly of entodermized ectoderm. Since entodermized ectoderm retains the same relations to other parts as when it was prospective ectoderm, questions whether it is inside out and which is its apical or anterior end arise and are discussed. It is suggested that the anterior end of the entodermized region may be the end connected with the remaining ectoderm, and it is pointed out that differential dye reduction supports this view. The small, thin-walled "neck," connecting remaining ectoderm and external entoderm in many forms with secondary modification

following the more extreme inhibitions, may perhaps be a stomodeum localized in the prospective anal region or a proctodeum. Development of the ciliated band in a circle about the flattened basal side of the remaining ectoderm after obliteration of ventrodorsality also suggests that a sufficient differential inhibition followed by recovery may bring this region into a condition somewhat resembling that of a ventral region.

7. Secondary modifications, opposite in direction from those of differential inhibition, result from differential tolerance or from conditioning (acclimation) to low lithium concentrations, and in more extreme degree from differential recovery after return to water, even from strongly inhibiting concentrations.

8. Secondary modifications after slight inhibition consist in relative enlargement of oral lobe and in increase in arm angle with short and small pluteus body. Excess of skeleton and supernumerary arms may also develop as secondary modifications, even in exogastrulae. The original prospective entoderm, inhibited to a solid cell mass and perhaps even after partial dissociation, is apparently able to regain, to some extent, its epithelial character during recovery. Entodermized ectoderm increases in size and may undergo further regional development, particularly in recovery. There is also evidence of re-ectodermization of the more apical levels of entodermized ectoderm.

9. In exogastrulae with entodermized ectoderm there is extensive reconstitution. The apical half or less than half of the original prospective ectoderm may develop arms or an arm and may approach pluteus form more or less, all parts except the extreme apical region developing from other than their original prospective regions. Entoderm of the more extreme exogastrulae may consist largely or wholly of entodermized ectoderm; but, if the original entoderm is not completely dissociated, it may form some part at the tip of the external entoderm, but much less than it would normally form and in harmonious development with entodermized ectoderm. In this case reconstitution involves production of less, instead of more, than normal. Entodermized ectoderm without original entoderm may develop three regions, separated by constrictions.

10. An appended review of the physiological gradient concept discusses questions of organization and its origin and points out that this concept does not deny existence of organization as an essential factor in development but is an attempt to throw some light on the problem of its origin.

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REGENERATIVE AND ACCESSORY GROWTHS IN PLANARIANS
II. INITIATION OF THE DEVELOPMENT OF REGENERATIVE AND ACCESSORY GROWTHS[1]

(Twelve figures)

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DURING the early part of the experimentation described in a previous paper (Goldsmith, 1939) animals with a supernumerary head in the mid-dorsal region were observed in stock cultures. At various other times individuals with one type of injury or another have been seen. Randolph (1897, p. 357) reported finding a specimen of *Planaria maculata* "with a cup-shaped growth on the back near the posterior end." It therefore seemed possible that in some cases the supernumerary structures had arisen from an injured region. A series of experiments was designed to test the ability of planarians to develop a supernumerary structure following an injury which does not completely separate two regions, and possibly by this means to add to our information on the phenomenon of induction.

In the vertebrates it has been shown that the action of the dorsal lip of the blastopore can be simulated by a considerable number of foreign tissues, both living and dead, and by specific chemical agents. It has also been demonstrated in a number of cases that nonspecific stimuli will evoke a definite structure, the nature of the structure depending upon the region which is stimulated.

For the invertebrates the following work should be noted. Browne (1909), using individuals of *Hydra viridis*,[3] concluded that any part of the *Hydra* body except the tentacle region could give rise to a new hydranth if properly stimulated. The stimulus, however, can be given only by material lying at the base of the tentacles or by similar tissue in a regenerated hydranth or bud. Rand, Bovard, and Minnich (1926) reported that peristome tissue of *Hydra oligactis* (=*fusca*) exerted "a stimulating or positive form creating dominance over column tissue." Child produced supernumerary hydranths in *Corymorpha* by means of single and radial incisions (1927, 1929) and by implants of tissue taken from various levels of *Corymorpha* stems (1929, 1935).

In flatworms the following observations are of interest as applied to the production of supernumary structures by local stimulation.

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[1] Revision and extension of a dissertation submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy at Harvard University.

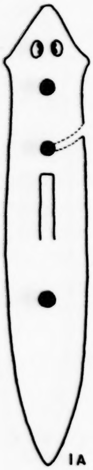
[2] I wish to express my thanks for the use of facilities in the departments of biology at Washington Square College, New York University, and at the City College of New York and for a grant from the Samuel Greenbaum Scholarship Fund of the City College of New York.

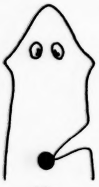
I take pleasure in acknowledging the helpful criticism and advice given throughout the course of this investigation by Professor H. W. Rand.

[3] Hyman (1930) points out that *Chlorohydra viridissima* (Schulze) (=*Hydra viridissima* Pallas, *H. viridis* Linnaeus), and *Pelmatohydra oligactis* (Pallas) Schulze (=*Hydra oligactis* Pallas = *Hydra fusca* Linnaeus).

Randolph (1897) "punctured" ten planarians "in the side with a needle." Her results were negative, in that 22 days after the operation seven remained alive and were typical in appearance.

Gebhardt (1926) transplanted regenerating planarian tissues from various body regions and found that the fate of the grafted regenerate depended upon its age and the differentiation it had undergone prior to transplantation. Goetsch (1929) transplanted head regenerates into the postcephalic regions of other planarians. The portion of the host body anterior to the implant was removed. The graft reorganized the remaining host body into what resembled a postcephalic region.





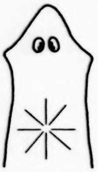


FIG. 1*A*.—The black areas indicate the regions to which the stimulus was applied. The broken line indicates laterial extension of the injury.

FIG. 1*B*.—Complete discontinuity resulting from lateral extension of the injury. Animals in which this occurred were discarded.

FIG. 2.—A series of "radial incisions."

Santos (1929, 1931) performed a series of homoplastic and heteroplastic transplantations in *P. maculata* and *Planaria dorotocephala*, each species serving (interchangeably) as host and donor. The cephalic ganglia, prepharyngeal, pharyngeal, and postpharyngeal regions were used as grafts and, with the exception of the ganglionic region, as sites of implantation. Santos concluded that a graft containing cephalic ganglia could act as an organizer or as a reorganizer, in that it may develop into a "head of some sort," which may bring about the reorganization of a region posterior to its level with the formation of a secondary pharynx and may completely reverse the polarity of a region anterior to its level. Miller (1938), using a modification of Santos' technique, successfully implanted cephalic grafts in the head, pharyngeal, and tail regions. Pieces of *Planaria gonocephala* containing cephalic ganglia and those from between the head and pharynx induced pharynges in the postpharyngeal regions of the host (Okada and Sugino, 1934, cited by Miller, 1938). Injection of planarian head extracts into the postpharyngeal region of individuals of *Euplanaria dorotocephala* produced no visible effect (Buchanan, 1938).

EXPERIMENTAL

Three different techniques were employed to ascertain whether a supernumerary structure could be produced in planarians by the application of a local stimulus: (1) radial incisions, (2) faradic stimulation, and (3) cautery. The animals were placed on a paraffin base, operated upon, and immediately returned to tap water. No anesthetic was used. Those cases in which the injury appeared to extend more than halfway through the parenchyma (dorsoventrally) and those in which the injury extended laterally so that complete discontinuity resulted (Figs. 1*A* and 1*B*) were discarded. In a number of successful cases in the cautery group only the superficial parenchyma was affected.

Since, of the three methods, the cautery procedure was found to give the greatest percentage of positive results, it was utilized to the greatest extent.

RADIAL INCISIONS

Incisions approximately 0.5 mm. in length, radiating from a center, were made with a cataract knife in individuals of *Dugesia tigrina*[4] and *Phagocata woodworthi* (Fig. 2). The incisions were made 1–3 mm. posterior to the eyes in animals ranging from 9 to 11 mm. in length.

Of about twenty-five specimens of *D. tigrina* so treated, several developed bulges at the site of injury after the wound region had closed completely. With the exception of two cases the elevated portion flattened out. One of the two developed into a small outgrowth, and the other into a small headlike structure containing what appeared to be typical eyes. The accessory head shifted its position by moving anteriorly so as to lie in close proximity to the original head.

Seventeen individuals of *Phagocata* were similarly treated. In six, pronounced bulges formed in the incised area after the wounds healed. One was resorbed, two developed into cylindrical outgrowths, one developed into an outgrowth with eyes, and two developed into outgrowths which finally became headlike and migrated anteriorly (Pl. I, Fig. 3).

FARADIC STIMULATION

A platinum electrode joined to an inductorium was touched to the dorsal surface of specimens of *D. tigrina*. The electrode was applied on the break, and the current was of an intensity sufficient to rupture the dorsal epithelium and to injure slightly the superficial parenchyma.

Twenty-five animals were so treated in the region 1–2 mm. posterior to the eyes, and twenty-one in the region posterior to the pharynx.

For the prepharyngeal series the following results were obtained: (1) one pronounced conical outgrowth which was resorbed after 10 days; (2) two slight bulges which developed into minute outgrowths and then pinched off at the base; and (3) one in which a single eye and another in which two small eyes developed at the site of injury.

In the postpharyngeal series one developed an elevation at the point where the electrode had been applied, and another formed a small outgrowth lateral to the site of the injury. The elevation subsided, and the outgrowth was resorbed.

CAUTERY

Bardeen (1901) "tried cutting planarians in two with a red-hot knife to see if cauterization would prevent the formation of tissue." The animals did not survive the operation.

The animals used in this group of experiments were *D. tigrina*, collected in the vicinity of Woods Hole and in Waverley, Massachusetts, and *Procotyla fluviatilis*[5] found in Waverley. The animals varied from 8 to 15 mm. in length.

A platinum electrocautery needle was heated slightly and was applied for a fraction of a second to various regions of the flatworm body (Goldsmith, 1932*a*, 1933). The greater number of experiments involved the dorsal aspect of the animal in prepharyngeal and postpharyngeal regions. Some of the planarians were burned in a region slightly posterior to the eyes; others slightly anterior to the pharynx; and others, slightly posterior to the pharynx (Fig. 1*A*).

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[4] In a private communication Dr. Libbie Hyman writes that the name *D. tigrina* (Girard) 1850 is the correct one. In previous papers I have used the name *P. maculata*. Other synonyms which occur in the literature are *Planaria lata*, *Euplanaria maculata*, *Euplanaria novangliae*, and *Euplanaria tigrina*. *Phagocata woodworthi* has previously been called *Phagocata gracilis* (Hyman, 1937).

[5] *Procotyla fluviatilis* has erroneously been called *Dendrocoelum lacteum*. *Dendrocoelum lacteum* is a European form and is not represented in America (Hyman, 1928).

After some practice in the use of the cautery, it was found that the area injured could be fairly well localized. If, within several days, no bulge appeared at the site of injury, the region was recauterized. The injured surfaces measured 0.2–1.0 mm. in diameter, those of the latter dimension occurring least frequently. In the cases in which the burned region was not circular in outline, the area would also fall within these limits.

Of the fifty cases of *Procotyla* operated upon, no bulges or outgrowths were obtained. The lack of pigment in the animal makes it difficult to determine the extent of the injury and the amount of tissue regenerated.

In the remainder of the work, *D. tigrina*, to which the subsequent statements apply (except where otherwise indicated), was used.

TABLE 1

THE FATE OF STRUCTURES PRODUCED BY THE CAUTERY OPERATION

Region Cauterized	Number of Cases	Number of Elevations	Number of Elevations in Which Eyes Did Not Develop and Which Subsided	Number of Elevations in Which Eyes Developed and Which Subsided with Eyes Remaining	Number of Elevations Developing into Outgrowths	Number of Elevations Developing into Outgrowths with Eyelike Pigment	Number of Elevations Developing into Outgrowths with Eyes (Curved Pigment Masses in Clear Areas)
Prepharyngeal....	181	60	18	9	11	1	21
Postpharyngeal...	169	54	36	0	13	3	2*

* In these two cases the anterior portion, including the pharyngeal region, of the animal was removed immediately after cautery.

RESULTS

The results obtained from similar injuries are not identical, and it was found that, in general, the tissue in the region anterior to the pharynx underwent further differentiation than did the tissue in the region posterior to the pharynx.

As a consequence of operations upon 181[6] animals in the prepharyngeal zone and upon 169 animals in the postpharyngeal region the structures obtained may be classed as follows: (1) supernumerary eyes; (2) bulges which persist for a few days or even several months without further differentiation and which at the end of these periods of varying duration are entirely resorbed; (3) bulges in which an eye or eyes develop and which are resorbed with the eye or eyes remaining in the body tissue; and (4) bulges which develop into outgrowths which (*a*) may pinch off a distal portion, and the proximal portion may be resorbed or else may give rise to a new distal region, (*b*) may form an aborted head, (*c*) may slowly shift their position so that they come to lie more and more anteriorly, and finally appear as if they were one of the members of a double head of the "fused" head type resulting from the splitting operation previously described (Goldsmith, 1939), or (*d*) may gradually become more definitely headlike but may persist in appearing as outgrowths from the dorsal surface of the animal. The data are summarized in Table 1.

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[6] These were the cases observed for varying periods of time. Several hundred others, in which the injury was too deep or extended too far laterally, were discarded.

In two cases structures which could be interpreted as supernumerary tails developed following the cautery operation in the postpharyngeal region. In one of the animals a small indifferent outgrowth developed at the site of the injury. This was resorbed. In the other, no outgrowth developed. In both cases the original tissue lateral and posterior to the region injured extended laterally and posteriorly, so that a structure, similar to a supernumerary tail arising from splitting the posterior portion of a planarian to one side of the mid-line, was produced (Fig. 4).

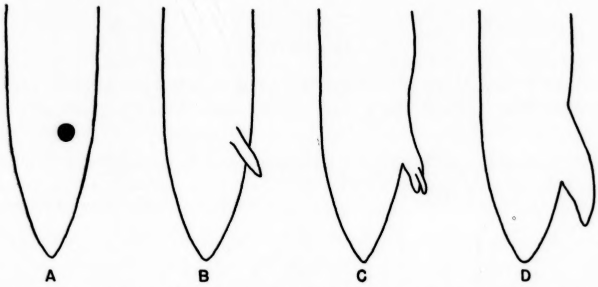

FIG. 4.—*A*, the black area indicates region to which cautery was applied. *B*, development of an outgrowth. *C* and *D*, the outgrowth diminishes in size and is finally resorbed. The original tissue extends laterally and posterially, so that a structure similar to a supernumerary tail produced by the splitting operation develops.

FORM OF OUTGROWTH

The outgrowths in general are somewhat conical or cylindrical and may have from one to five eyes (Fig. 5). Where the eyes are more than two, they are usually distributed over the anterior, posterior, and lateral aspects of the growth. All surfaces are similar in pigmentation to the dorsal surface of the animal, from which the outgrowth arises.

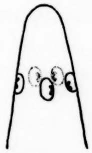

FIG. 5.—Diagrammatic outline of an outgrowth with five eyes produced by cautery.

Double outgrowths, such as those shown in Plate I, Figure 6, may develop. Outgrowths of this type appeared as if they were composed of two heads (each having a set of eyes), which had fused by their ventral surfaces. These structures usually developed at a site which had been cauterized more than once. It is possible that subsequent cautery operations involve not quite the same zone as the previous operations. If two active centers are set up, the "double" outgrowths may result.

MIGRATION

The outgrowths exhibited the tendency to migrate anteriorly and laterally, so that a structure originally slightly anterior to the pharynx may finally come to lie between, or lateral to, the eyes of the host (Pl. I, Figs. 7, 8, and 9).

Santos (1931) observed this tendency in postcephalic grafts. The direction of movement here depended upon the orientation of the transplant, so that a graft in reversed anteroposterior orientation may migrate with its anterior end leading, moving farther and farther from its original body-level. Miller (1938) has also noted this migratory tendency of grafts.

Migration of grafts in *Hydra* have been reported by Rand (1899) and by Hefferan (1902).

RESORPTION

It has been observed that an elevation and even a small outgrowth arising as a result of a local stimulus may persist for a short time and then be gradually resorbed. The resorption time varied greatly. In one case the induced outgrowth was small and was resorbed within 25 days after the cautery operation; in another, the outgrowth remained for 70 days; and in a third, resorption was complete after 4 months. In the animal shown in Figure 10 resorption did not begin until 11 months after the initial appearance of the outgrowth. A month later only the eyes remained (Fig. 10). In a similarly treated planarian the outgrowth gradually decreased in size but was still present 13 months after its origin. It should be pointed out that in many cases the outgrowths have remained and have maintained their size and activity (Pl. I, Fig. 11).

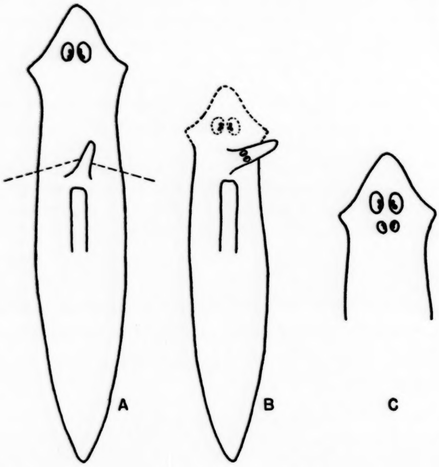

FIG. 10.—*A*, a conical outgrowth developed after cautery. The anterior portion of the body is removed (incision indicated by broken line). *B*, eyes developed in the outgrowth and a head is regenerated. *C*, the outgrowth has been resorbed, but its eyes remain.

In connection with this resorption of outgrowths the utilization of a supernumerary eye in the formation of a typical head was observed. In this case a small mound of tissue developed after cautery of the region slightly posterior to the eyes (Fig. 12, *A*). Nine days after its appearance the mound subsided, and an eye which had developed in it remained. The original head was removed by a transverse cut posterior to the original eyes (Fig. 12, *B*). Tissue which shaped up as a typical head regenerated anterior to the remaining eye (formerly the supernumerary one). Its vesicle increased in size, and an additional pigment cup developed in it. A new left eye was formed (Fig. 12, *C*).

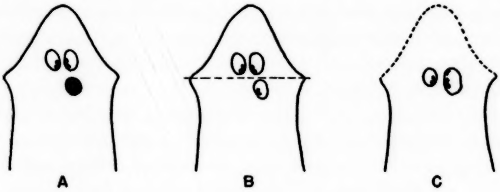

FIG. 12.—*A* and *B*, development of a supernumerary eye following cautery. The original head is removed, as indicated by broken line. *C*, a head with a left eye is regenerated. In the development of the right eye the supernumerary one is utilized.

DISCUSSION

The observations recorded in this paper demonstrate the ability of several types of injury to evoke a response which may culminate in a headlike structure. They further indicate that the degree of differentiation which the structure may undergo is, in part, dependent upon the region in which the operation is performed. It was found that structures in prepharyngeal regions developed headlike morphological characteristics which, with two exceptions,[7] did not obtain for those in the postpharyngeal area. Since no foreign tissue was involved, it can be definitely stated that the tissue of the injured animal has differentiated to form the new growth.

Gebhardt (1926), Santos (1929, 1931), and Miller (1938), by grafting pieces of planarians, produced outgrowths very similar in appearance to those described in this paper. Santos concluded that the ganglionic graft is an organizer in the sense of Spemann. In its new location the graft develops into a headlike structure and then induces an outgrowth from the host body (part of the outgrowth appears to rise from donor tissue). The sequence of events in my experiments is quite different. The injured region becomes elevated. This elevation increases in size, so that it protrudes markedly; and eyes and auricles may develop in the distal portion of the structure so formed.

A similar phenomenon is recorded by Child (1927) for *Corymorpha*, where "radial incisions" served to produce a secondary hydranth.

The behavior in the injured planarians and of the *Corymorpha* individuals described by Child would then necessitate shifting the emphasis from the agent utilized (injury or implantation of tissue) to the zone to which the agent is applied or transplanted. This concept is in harmony with the facts of induction derived from the work on Amphibia.

Supernumerary limbs, composed of host tissue, developed following the implantation of otic vesicles (Balinski, 1927*a*), nasal placodes (Glick, 1931), tailbud (Goldsmith, 1932*b*), and celloidin (Balinski, 1927*b*). Implantation of foreign tissue or irritation of the middorsal line region of *Triton* induced a supernumerary crest of host tissue (Milojevic, Grbic, and Vlatovic, 1926). The experiment of Guyénot and Schotté (1927), in which a regenerating limb bud with incipient digits formed "a small characteristic tail" when transferred to the side of a *Triton* tail, and Gebhardt's work with planarian regenerates further impresses one with the importance of the so-called "morphogenetic field."

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[7] Immediately after cautery the entire anterior portion, including the pharyngeal region, of the animal was removed.

Is the difference in the behavior of the prepharyngeal and postpharyngeal regions following radial incisions, faradic stimulation, and cautery in flatworms to be attributed to differences of the fields? The answer to this may be approached by roughly plotting the sequence of events after injury.

A region is injured. It is repaired by the addition of new tissue. The new tissue may do more than replace the portion lost or injured; it may continue to grow. There is now present something akin to the regenerates used as transplants by Gebhardt, but, differing in that, it is an "indifferent" regenerate, since it has formed at neither the head nor the tail end of a worm. Now if the injured region is a prepharyngeal one, the outgrowth may develop eyes and auricles; if it is the postpharyngeal region which has been irritated, the outgrowth will usually continue to be indifferent. This is shown by the data in Table 1.

Here we are dealing with the "host" alone. There is no donor tissue. It is realized that tissues, such as those of the nervous and digestive systems, will be exposed at the point of injury. In a review of the literature (Goldsmith, 1939) it was shown that at present we cannot regard the digestive influence as a formative influence in head regeneration. The influence of the nervous system in this connection is still not clear. Beyer and Child (1930) conclude that the nervous system may be a localizing influence but is not a determining factor in the formation of a head. Goldsmith (1939) found that in many cases head regeneration failed to take place although the lateral nerves were present. Further, it must be remembered that the exposure of the tissues occurs in both the prepharyngeal and the postpharyngeal regions. In view of the foregoing, it might appear that the potentialities of the two territories differ greatly, the prepharyngeal being able to form a head and the postpharyngeal being unable to do so. However, since a decapitated prepharyngeal piece and a piece from which the entire pharyngeal region has been removed will behave similarly in that both will regenerate heads, we must concede that such cannot be the case. That there is some difference between these regions cannot be denied, but the difference must be one of degree rather than of kind. That this latter view may approach the correct interpretation is in part borne out by cytological studies.

FORMATIVE CELLS

"Bildungszellen," regarded as the precursors of regenerating organs, were described in the parenchyma of rhabdocoeles by von Wagner (1890). Keller (1894) stated that the number of these cells varied directly with the regenerative power of the species.

In individuals of *P. maculata* dividing by fission the number of formative cells is greatly increased in the region of the body dorsal to the gut, so that they become the most numerous of the mesodermal constituents (Curtis, 1902). Additional work (Weigand, 1930; Curtis and Schulze, 1934) has led Curtis (1936) to regard the formative cells in planarians as those functioning in the formation of new parts during regeneration. However, "the immediate origin of these cells is not so obvious." Child and Watanabe (1935) are of the opinion that, following removal of an anterior region, partially differentiated cells (not undifferentiated formative cells), owing to a change in their "physiological environment," become embryonic and may then give rise to a new region. Curtis and his collaborators have made a comparative study of the number and distribution of the formative cells in normal and irradiated individuals of *P. maculata*, *Planaria agilis*, and *Procotyla fluviatilis*. The number of formative cells in the former two which exhibit marked powers of regeneration is considerably higher than in *Procotyla*, which has a low regenerative capacity (Lillie, 1901; Morgan, 1904; Isely, 1925). Cell counts made in individuals of *P. maculata* from a stock in active fission show that the number of formative cells mounts rapidly, beginning at a point slightly posterior to the eyes, reaching a maximum at a point midway between the eyes and the anterior extremity of the pharynx. The number then decreases gradually until the pharyngeal region is reached, where an abrupt diminution is observable. The number then remains low, until the level of the second zoöid is reached. Here there is a second increase, followed by a sharp decline in posterior tail region. *Procotyla*, like *P. maculata*, contains more formative cells in its prepharyngeal region than in the postpharyngeal, but the absolute number is lower (Curtis and Schulze, 1934).

Table 1 shows clearly that the differentiation attained by anterior structures is greater than that attained by posterior ones (two exceptions; in these the entire anterior region, including the pharyngeal portion, had been removed at the time of cautery). The outgrowths arising in the regions corresponding to those said to be highest in formative cell content (Curtis and Schulze, 1934) are those in which eyes develop and which in some cases take on a headlike appearance. It was in this zone that the incidence of head regeneration was highest following removal of one head in a double-headed planarian (Goldsmith, 1939).

In a number of instances where the cautery operation was followed by wound healing but no outgrowths, repetition of the operation gave positive results; in one case an outgrowth developed only after the sixth cautery operation.

Flexner (1898) noticed that, following transection in *Planaria torva*, there is a marked production of new cells. These Curtis (1902) concludes are dividing formative cells. If these observations and their interpretation are correct, then by successive injuries one should be able to increase the number of formative cells per unit volume. This the successive cautery operations may have done, and consequently an outgrowth was formed.

From this it follows that every point along the anteroposterior axis of individuals of *D. tigrina* should respond similarly, were the stimuli sufficient in number and the intensity of the stimuli sufficient in degree.

The negative results following the cautery operation in fifty individuals of *P. fluviatilis* may be related to the smaller number of formative cells in *Procotyla*. Curtis and Schulze (1934) give the approximate ratio as ". . . . 8.5 formative cells in *P. maculata* to 1 in *Procotyla fluviatilis* for equal unit areas in similar regions of the two worms."

If there is a relationship between the number of formative cells and the degree of differentiation which structures may undergo, can the nature of the relationship be defined in chemical terms?

Sulfhydryl compounds.—The glutathione content in *Procotyla* is low, while that of *P. agilis* is high; further, the glutathione content is higher in regenerating tissues. These facts Coldwater (1933) correlated with the "formative cell" count. Coldwater, while showing that $^{-}$SH compounds accelerated the rate of regeneration of posterior segments of *Tubifex*, was unable to produce a similar effect in *P. maculata* or *Procotyla*.

Recently, Owen, Weiss, and Prince (1938) found that glutathione (but not cysteine) stimulated regeneration in sectioned planarians and the fission-rate in whole animals. Similar effects were observed after treatment with 1:2:5:6-dibenzanthracene. Reimann and Hammett (1935) believe that the stimulating action of this, as well as of other compounds, may be exerted through the $^{-}$SH group.

In a previous report (Goldsmith, 1934) the belief was expressed that the so-called "inductions" produced by various agents arose as a result of irritation. If this were true, then cautery, as used in the flatworms, should also act as an "evocator" when used on other organisms and should awaken potencies lying dormant. This has been confirmed by Cohen (1938), who induced various structures in *Rana pipiens* by inserting a microcautery needle into the blastocoele of the early gastrula. It was also pointed out (Goldsmith, 1934) that injury may release sulfhydryl[8] (scratching a growing root tip resulted instantly in a freeing of sulfhydryl [Hammett, 1929]), which may accelerate cell division in those cells which are the relatively undifferentiated ones, the formative cells in planarians. Not only do the formative cells proliferate following an injury, but they differentiate; and the height of differentiation in planarians is expressed in head formation. It must be remembered that there is an inhibitory influence emanating from the anterior end of the animal (cf. Child, 1929; Goldsmith, 1939; and Weiss, 1939, pp. 380–82). The degree of differentiation which the developing structure will undergo will depend upon the ratio of the regenerative capacity of the injured region to the inhibitory action exerted by the apical end of the worm.

SUMMARY

1. Supernumerary eyes or small outgrowths with or without eyes may arise in individuals of *D. tigrina* (syn. *P. maculata*) following faradic stimulation.

2. Supernumerary structures, which in some instances develop into heads, may arise as a result of radial incisions (*Phagocata* and *D. tigrina*) or cautery (*D. tigrina*). In most cases the structures arising after injury to the prepharyngeal region differentiate to a greater extent than do those arising after injury to the postpharyngeal region. The similarity between these outgrowths and those produced by the implantation of planarian tissues is pointed out.

3. Repeated applications of the cautery needle may result in the development of a supernumerary outgrowth after previous applications (five in one case) had been unproductive.

4. In a number of cases the outgrowths migrated anteriorly and laterally, so that a structure originally slightly anterior to the pharnyx may come to lie between or lateral to the eyes of the "host."

5. The outgrowths may persist for variable periods of time and then be gradually resorbed. Eyes which had developed in these structures remain as supernumerary eyes in the "host" tissue long after the induced growths have been resorbed.

6. Individuals of *Procotyla* reacted negatively to all the stimuli.

7. It is suggested that the high degree of differentiation attained by the outgrowths induced in the prepharyngeal region in *Dugesia* and the negative results in *Procotyla* may be correlated with the "formative cell" distribution. These in turn may be related to the presence or release of sulfhydryl.

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[8] Although a mass of data in support of the formative cell $^{-}$SH concept is available, the evidence is not entirely in its favor; and we must be careful, in the present state of knowledge, not to regard the $^{-}$SH group as omnipotent.

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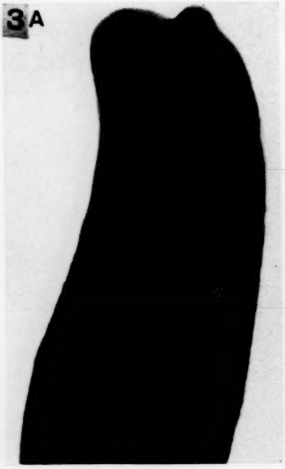
PLATE I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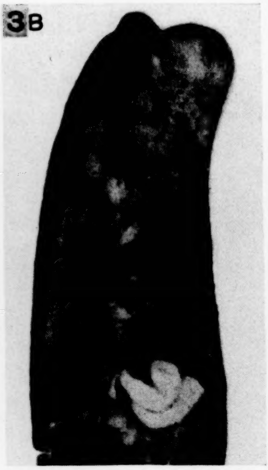
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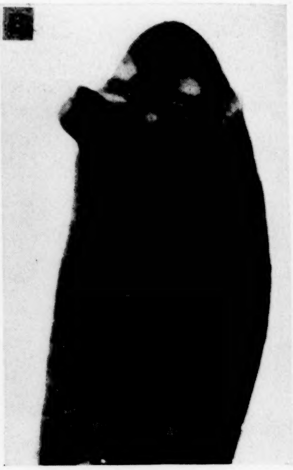
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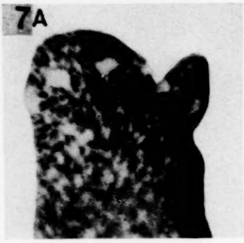
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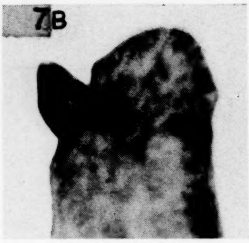
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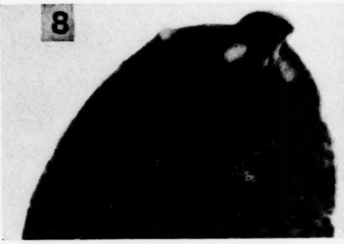
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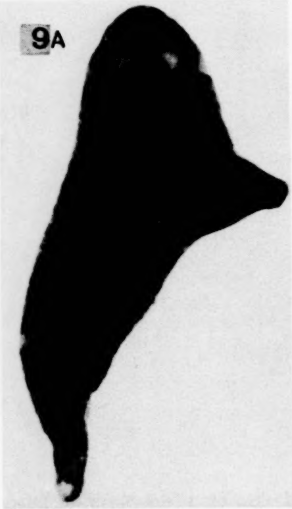
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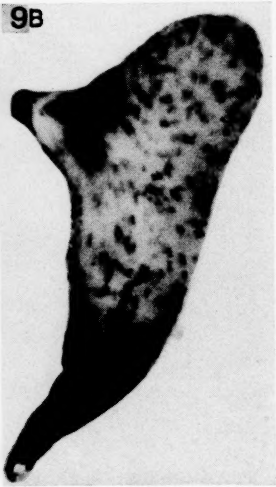
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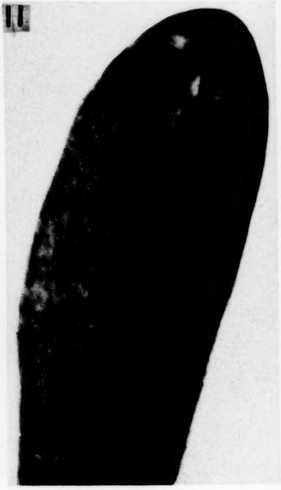
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PLATE I

FIG. 3*A*.—A dorsal view of an individual of *Phagocata* showing the supernumerary head (to the right of the original head) which developed following "radial incisions."

FIG. 3*B*.—A ventral view of the animal shown in Fig. 3*A*.

FIG. 6.—An outgrowth possessing two sets of eyes, one pair on the anterior face and another on the posterior face. The pigment cups in the clear areas (vesicles) do not show in the photograph. An additional structure arises from the left side of the outgrowth.

FIG. 7*A*.—A dorsal view showing a supernumerary head which had migrated from the mid-dorsal, prepharyngeal region.

FIG. 7*B*.—A ventral view of the animal shown in Fig. 7*A*. It is clear that the supernumerary head arises from the dorsal side.

FIG. 8.—A supernumerary head lies directly posterior to the eyes of the original head. Many of the outgrowths migrate anteriorly, as did this one.

FIGS. 9*A* and 9*B*.—A dorsal and ventral view of a supernumerary head which had migrated laterally.

FIG. 11.—An outgrowth (15 months after its initial appearance) arising from the dorsal aspect of the animal. One vesicle is visible. The pigment cup in it cannot be seen. Another eye is present on the other side of the outgrowth.

The supernumerary structures shown in Fig. 6–11 were produced by cautery.

SOMATIC EFFECTS OF TEMPERATURE ON DEVELOPMENT IN DROSOPHILA MELANOGASTER. I. PHENOCOPIES AND REVERSAL OF DOMINANCE

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MANY investigators of the effects of high temperature on various stocks of *Drosophila melanogaster*—Goldschmidt (1929, 1935), Jollos (1930, 1933), Plough and Ives (1932, 1935), and others—have noted among the imagoes heated in prepupal stages individuals which phenotypically resembled genotypes other than the ones used. These "phenocopies" (Goldschmidt's term) included stubby, forked, extra, and missing bristles; rough, and deformed eyes; straplike, balloon, spread wings; certain eye and body colors; and many other characters. In short, representatives of nearly every type of mutant character were obtained. The data, particularly those of Goldschmidt, indicated that the extent and type of the effect were dependent on four factors: (1) the temperature used, (2) the duration of the treatment, (3) the genetic constitution of the treated flies, and (4) the developmental age of the flies at the time of treatment.

In 1935 a series of investigations was started in the Amherst laboratory to determine more precisely the various factors involved, particularly the importance of the genetic constitution and the age of the flies at the time of treatment. An inbred wild stock, Florida +10, and F_1 heterozygotes of this stock against multiple mutant strains representing the first, second, and third chromosomes were used in these experiments. It was expected that the heterozygotes would be more sensitive than the wild-type flies to the effects of temperature. At the same time, effects might be visible which would be masked by the still greater effects of genes in homozygous condition.

In these experiments the heterozygotes and the wild-type flies were subjected to a 12-hour exposure to $36\overset{\circ}{.}5 \pm 0\overset{\circ}{.}2$ C. at definite periods during larval and pupal development. The control temperature was held at $25\overset{\circ}{.}3 \pm 0\overset{\circ}{.}2$ C. Egg-laying periods were from 2 to 6 hours in duration.

The phenocopies obtained in these experiments represent a wide range of types. The characters most frequently found include: alterations in size and form of eyes, legs, wings, bristles, and abdomen; presence or absence of specific wing veins and body bristles; and modifications in eye and body color. These and other phenotypes provide numerous close parallels to the known series of mutant characters involving such effects. Thus, the phenotypes appearing after temperature change during development are often similar to those produced by mutant genes acting when the temperature is constant; or, stated in another way, an individual of one genetic constitution raised at one temperature resembles phenotypically an individual of a different genetic constitution raised at another temperature.

The data included in Tables 1–4 were selected to demonstrate the phenotypic expression of the nominally recessive genotypes of the treated stocks. The results indicate the percentage of flies showing the particular character produced following heat treatment at a specific time in development. The incidence of two or more phenotypic characters in the same fly is not tabulated, since a significant correlation between such characters was indicated in only one case, i.e., the connection between truncate and curved mentioned below.

OBSERVATIONS

As Goldschmidt (1935) and others have already observed, the time at which certain phenocopies may be induced is very specific. Inspection of the data in Tables 1–4 shows that the temperature-effective periods for different characters are not of the same duration and may occur at different periods in development. For example, eye shape seems to be thermally sensitive during most of the larval life-history, whereas certain wing and bristle characters respond to temperature treatment only at quite specific periods in early pupal life. Furthermore, the time of appearance of many phenocopies agrees closely with the facts already known about the ontogeny of *Drosophila*. Since the time during which puparium formation takes place at 25°3 C. was found to be from 4 to 5 days for +10 and +10/2ple flies, in the subsequent analysis 4.5 days is used as the time of puparium formation.

TABLE 1

PHENOTYPIC EFFECTS ON WILD-TYPE STOCK (FLORIDA +10) OF EXPOSURE FOR 12 HOURS TO 36°5 C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye	0.4	10.0	45.4	60.0	10.0	25.6	1.8	3.1
Short wing	0	4.0	9.1	13.3	2.0	3.8	0	16.7
Curved wing	0	0	0	0	0	2.6	0	15.6
Truncate wing	0	0	0	0	0	1.3	0	0
Vortex	0	0	0	0	0	0	0	0
Plexus	0	0	0	0	0	0	0	0
Crossveinless	0	0	0	0	0	0	0	0
Forked bristles	0	0	0	0	0	0	0	32.9
Short bristles	0	0	4.5	3.3	2.0	10.3	10.9	16.7
Missing bristles	0	0	0	10.0	4.0	15.4	12.7	3.1
Microchaetes gone	0	0	0	3.3	20.0	0	3.6	10.9
Hairy thorax	0	0	0	0	4.0	0	0	0
Dark body	0	0	0	0	2.0	0	0	0
Total number of flies observed	235	50	22	30	50	78	55	64

Among the phenocopies most specific in time of production are those affecting the appearance of the wings. Most of the wing characters were induced by treatment after the time of puparium formation. The data indicate that the sensitive period for truncate begins approximately at the start of actual pupation. The vein effects have also been localized largely in the first 12 hours after pupation. This conforms with the data of Hersh and Ward (1932) and Stanley (1935) on the temperature-effective period for

TABLE 2

PHENOTYPIC EFFECTS ON +/1PLE (SCUTE, CROSSVEINLESS, VERMILION, FORKED) OF EXPOSURE FOR 12 HOURS TO 36°5 C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye	0.5	7.7	64.3	77.0	18.6	22.2	0	0
Short wing	0	0	43.0	0	0	0	0	16.7
Curved wing	0	0	0	0	0	0	0	0
Truncate wing	0	0	0	0	0	0	0	0
Vortex	0	0	0	0	0	0	0	0
Plexus	0	0	0	7.7	14.0	11.1	32.0	0
Crossveinless	0	0	0	0	7.0	1.8	8.0	0
Forked bristles	0	0	0	0	9.3	3.6	0	37.5
Short bristles	0	0	0	15.4	7.0	1.8	20.0	31.2
Missing bristles	0	0	50.0	46.0	18.6	31.4	28.0	8.3
Microchaetes gone	0	0	0	0	18.6	7.4	20.0	2.1
Hairy thorax	0	0	0	0	0	0	0	0
Dark body	0	0	7.1	0	0	0	0	0
Total number of flies observed	216	13	14	13	43	54	25	48

TABLE 3

PHENOTYPIC EFFECTS ON +/2PLE (DUMPY, BLACK, PURPLE, CURVED, PLEXUS, SPECK) OF EXPOSURE FOR 12 HOURS TO 36°5 C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT								
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.25	5.5
Rough eye	0.9	32.0	57.6	36.6	6.4	15.8	27.2	44.4	1.2
Short wing	0	4.4	11.5	3.8	2.1	1.6	1.6	6.2	57.5
Curved wing	0	0	0	0	2.1	3.2	1.6	3.7	62.4
Pointed wing	0	0	0	0	0	0	9.3	14.6	0
Truncate wing	0	0	0	0	1.1	1.6	23.4	23.4	0
Vortex	0	0	0	0	0	0	1.3	2.5	0.6
Plexus	0	0	0	0	16.0	3.2	37.6	42.0	3.2
Crossveinless	0	0	0	0	2.1	0	2.6	3.7	0
Forked bristles	0	0	0	0	13.8	0	7.8	9.9	55.1
Short bristles	0	0	0	5.1	2.1	0	0	0	4.9
Missing bristles	0.2	4.4	11.5	25.4	1.1	9.5	4.9	5.0	1.8
Microchaetes gone	0	1.5	0	0	28.7	4.8	28.9	33.3	1.8
Hairy thorax	0	0	0	0	0	0	0.7	1.2	0
Dark body	0.7	8.7	0	13.9	22.4	6.4	2.6	3.7	7.8
Total number of flies observed	439	69	26	79	94	63	308	81	165

normal wings and with the period during which wing phenotypes were found by Goldschmidt, Jollos, and Plough and Ives. The embryological evidence also fits in. Although Auerbach (1936) has localized the period of growing-out of the wing pouch in the mature larva and prepupa, the morphogenetic effect of the mutant dumpy, i.e., truncate wing, first becomes visible from 18 to 36 hours of pupal age (Auerbach, 1936; Goldschmidt, 1937).

Short wing also seems to have a temperature-effective period at 3.5–4.0 days. Auerbach has found that the mesothoracic buds divide into the separate thoracic and wing disks at this time, i.e., in the early part of the third instar. She believes that vestigial, whose temperature-effective period begins at this time, acts during the third instar by effecting a reduction in the size of the wing disk. A similar explanation may account for the early sensitive period for short wings.

TABLE 4

PHENOTYPIC EFFECTS ON +/3PLE (ROUGHOID, HAIRY, SCARLET, PEACH, SPINELESS SOOTY) OF EXPOSURE FOR 12 HOURS TO 36°.5 C.

(Data calculated in percentages)

PHENOTYPES OBSERVED	AGE IN DAYS AT TIME OF TREATMENT							
	Control	3.0	3.5	4.0	4.5	4.75	5.0	5.5
Rough eye	0.6	94.4	90.0	83.4	7.9	8.4	13.6	7.0
Short wing	0	1.9	22.5	33.3	0	5.0	0	21.9
Curved wing	0	0	0	0	0	0	1.2	0
Truncate wing	0	0	0	0	0	0	0	0
Vortex	0	0	0	0	0	0	0	0
Plexus	0	0	0	0	0	0	0	0
Crossveinless	0	0	0	0	0	0.8	0	0
Forked bristles	0	0	0	0	0	0	0	14.1
Short bristles	0	0	2.5	33.3	1.3	8.4	43.2	26.3
Missing bristles	0	0	12.5	16.6	10.5	12.3	17.3	0
Microchaetes gone	0	0	0	0	14.5	1.6	7.3	7.0
Hairy thorax	0	0	0	0	3.9	0	0	0
Dark body	0.3	24.1	25.0	16.6	30.2	8.4	17.3	17.6
Total number of flies observed	318	54	40	6	76	121	81	114

The sensitive period for rough and abnormal eyes seems to extend throughout the period studied. The period of greatest effect, however, appears to lie in the third instar, before the formation of the ommatidia, which takes place at the end of the larval period (Krafka, 1924; Chen, 1929; Medvedev, 1935).

The bristle and hair effects occur mainly in the postlarval groups. However, the sensitive period for extra bristles lies in the 3.5–4.0-day interval, while the temperature-effective period for missing bristles apparently extends throughout the observed periods with a peak at 4.0 days and a lesser one at 5.0 days. The prepuparial effect on missing bristles agrees with the data of Child (1935) and of Ives (1939) on the temperature-effective period for scute. The bristle and hair effects, considered *in toto*, correspond in point of time with the evidence of Plunkett (1926) and Goldschmidt (1935). Robertson

(1936) has found that the trichogenic cells do not develop until 27 hours, and the bristles and hairs not until 30 hours after puparium formation.

Abnormal legs, most of which were characterized by crippled tarsae of the third pair, occurred largely in the 3.5- and 4.0-day period. These effects, resembling some phenotypes of crippled (Komai, 1926) were induced, as were those of Hoge (1915), by temperature before the morphogenesis of the legs, which takes place in the first 4 hours after puparium formation (Auerbach, 1936; Robertson, 1936).

INTERPRETATIONS

The occurrence of these phenocopies may be interpreted in terms of the rate theory of development. According to the current rate theory, the characters of an organism are determined by the rates and interactions of developmental processes, the rates and durations of which are modified by the interaction of genetic and environmental factors during development.

Variations in the developmental environment of the wild genotype produce only slight changes in the wild phenotype. Wild-type flies raised within the range of normal temperature show slight differences in facet number (Margolis and Robertson, 1937), wing size (Stanley, 1931, 1935; Hersh and Ward, 1932; Riedel, 1934), and other characters. At the extremes in the temperature range these differences are more accentuated. This suggests that the temperature coefficients of all the developmental processes in the wild type are not equal. The temperature coefficients, however, harmonize sufficiently to produce a fairly uniform phenotype. This comparative constancy has been explained by Plunkett (1932) as due to a natural selection of genotypes which will produce uniform phenotypes under ordinary conditions, i.e., within the normal range of temperature, humidity, and other environmental factors affecting the development of organism.

Variations in the developmental environment of a mutant genotype, however, produce marked changes in the mutant phenotype. The extent of the effect varies with each mutant genotype (Bar, vestigial, scute, and Dichaete series of alleles, abnormal abdomen, extra legs, etc.) and also with the type and extent of the environmental change (temperature, humidity, and food). With respect to the temperature effect, it may be supposed that the temperature coefficients of certain reactions, affected by the mutant gene or its products, differ markedly from the temperature coefficients of development as a whole. This differential effect on the rates of developmental processes produces different effects with variations in temperature. Varying food conditions, humidity, and other factors affect differentially the time of development (as a whole) and the duration of processes affected by the mutant gene, thus producing recognizable phenotypic differences.

A mutant, moreover, may be considered as a wild genotype with a single gene substitution. This genic variation produces an effect like a change in the external environment. The extent of its effect may be supposed to depend upon (1) the rates and interactions of the developmental processes affected by the mutant gene as compared to the effect of the wild-type gene on these processes; (2) possible effects of the mutant gene on reactions not affected by its wild-type allele (and the reciprocal of this); and (3) the temperature coefficients of the reactions affected by the mutant gene as compared to the wild-type gene. When considering the action of genes in development, then, the factors involved are rates and temperatures coefficients of developmental processes. Variations in genotype may be supposed to be similar to variations in environmental conditions in so far as both affect the rates of developmental processes.

On the basis of this reasoning, short exposures of the wild type to high temperatures outside the range of the "normal" environment, but not long enough to kill the organism, should have a differential effect on the reactions going on at that time. The developmental processes may be expected to be thrown out of step with one another because of their different temperature coefficients. The phenotypic effect of this exposure, as mentioned previously, depends upon at least four variable factors: (1) the genotype, i.e., the rates and temperature coefficients of the reactions affected; (2) the intensity of the treatment, i.e., the temperature used; (3) the duration of the treatment; (4) the time of treatment, i.e., the reactions affected. The end-products produced by these reactions may differ quantitatively or qualitatively from end-products produced under normal conditions. These end-products may be the adult character, or they may be substances which will affect the reactions occurring at a later period in development.

It then follows that exposures of the wild type at certain periods may be expected to produce phenocopies. The results of these experiments show further that an effect on a character may be produced before its morphological ontogenetic processes appear; e.g., bristle effects may be produced by treatment during the early larval period (Tables 1–4), although the bristles are not laid down until more than a day after puparium formation. Ives (1939) has shown that similar effects may even be produced by treatment during the embryonic period. This may be interpreted as a temperature effect on chemico-embryological processes, which in turn determine the rates of visible reactions ensuing at a later period in development. This significant concept, illustrated by Wright (1934) and Powsner (1935), appears to have been overlooked by many workers who emphasize the latter type of effect, viz., on the visible processes. Other effects, e.g., the truncate wings, appear to be specific for the morphological developmental period of the organ.

Reversal of dominance.—As stated before, the data included in Tables 1–4 were selected to demonstrate a particular aspect of the production of phenocopies, i.e., the phenotypic expression of the nominally recessive genotypes of the treated stocks. This phenomenon is best described as a reversal of dominance. Although rough eye occurs in all the stocks, its percentage of manifestation is the greatest in heterozygous 3ple, which includes roughoid (ru). Shortening of the wing may be an effect of the second chromosome gene, dumpy (dp). When treated in the postlarval temperature-sensitive period, short wing is more than twice as frequent in +/2ple as in the other stocks; fully half of the flies in the former group show the phenotype. After treatment in the larval period, the manifestation of this character is greatest in +/1ple and +/3ple. This suggests that there is another critical period independent of that acted on by the dumpy gene.

Except for some cases in +10 stock, curved-wing phenotype is manifested only in +/2ple, which includes curved wing (c). It should be added that curvature of the wing is often associated with extreme dumpy. Truncated wing is practically unique in heterozygous 2ple stock. Pointed wing, which refers to an oblique flattening of the inner distal end of the wing, seems to be a slight truncate effect. Vortex, a pleiotropic effect of dumpy, also occurs only in the presence of that gene. Plexus, the presence of extra bits of vein in the distal wing cells, is of variable occurrence both as to position and extent. The proportion of +/2ple flies which shows this character is high (42 per cent), as might be expected from the presence of plexus (px); but a smaller percentage (32 per cent) of +/1ple flies also shows the character. A partial loss of the posterior crossvein was observed in +/1ple (8 per cent), which includes crossveinless (cv), and to a lesser degree in +/2ple (3.7 per cent). The fact that plexus and crossveinless appear in the presence of both cv and px suggests a close interrelation between the action of these genes.

The bristle effects do not lend themselves so readily to analysis. The percentage of forked bristles in +/1ple (37.5 per cent), which includes forked (f), is almost equaled in +10 (32.9 per cent) and is exceeded in +/2ple (55.1 per cent). Short bristles, an effect of spineless (ss), are most frequent in +/3ple (43.2 per cent), followed by +/1ple (31.2 per cent). Missing bristles, characteristic of scute (sc), are found primarily in +/1ple (50). The percentage of missing microchaetes seems to bear no relation to the presence of bristle-removing genes.

Dark body occurs in both +/2ple (22.4 per cent) and +/3ple (30.2 per cent). Trident is included with dark body in the data. The greater effect of sooty in chromosome 3, as compared with black in chromosome 2, is paralleled by the higher percentage of manifestation in +/3ple.

In all these cases the effect was variable in extent, suggesting different degrees of reversal of dominance. Crossveinless varied from a slight deficiency to an almost complete lack of the crossveins. A truncate series was obtained with all degrees from normal through a phenotype resembling that of the oblique allele to an effect as great as that of homozygous dumpy raised at 25° C. Vortex varied from slight whorling of microchaetes to distinct humps on the thorax.

DISCUSSION

From the foregoing it is apparent that a gene normally recessive, such as dumpy or plexus, may behave as a dominant under certain other environmental conditions. The degree of dominance of a gene is, therefore, a function of the environment within which the developmental processes take place. This concept is a corollary of the rate theory.

In the developmental processes leading to character expression both alleles of a gene (if both are present) affect the character. This has been well established for the color genes of rodents (Wright, 1927; and others), the vestigial alleles of *Drosophila* (Mohr, 1932; Goldschmidt, 1935; Harnly, 1936), the scute series (Serebrovsky, 1930; Dubinin, 1932; Child, 1936), and for many other genes and characters in plants and animals. It is self-evident for two mutant alleles, e.g., scute 1/scute 5, vg^{nw}/vg^{ni}, etc. It is not as apparent in the case of scute/wild type or vg/wild type, etc. The greater effect of the wild-type gene on the developmental processes hides the effect of the mutant gene, giving rise to the phenomenon of dominance. That the recessive gene does produce an effect, however, has been shown by Riedel (1934) for vestigial/wild.

On this basis the heterozygote and homozygous wild type should behave differently with changes in the external environment (Lutz, 1913; Hersh and Ward, 1932; Riedel, 1934; Stanley, 1935; Margolis, 1935). The effect of very high temperature should be even more striking because of the greater differences in the rates of the developmental processes at these temperatures. Normal dominance relations, dependent upon "normal" environmental development, should be upset by heat treatment at particular periods in development. These periods should, furthermore, coincide with the periods at which the homozygous wild type is affected (since presumably the same developmental reactions are being affected). The data presented show that this has been realized in these experiments. "Dominance has been reversed" for a number of genes.

SUMMARY

1. Phenocopies representing most of the known types of mutant characters in *Drosophila* have been obtained by subjecting wild stock and F_1 heterozygotes of this stock against multiple mutant strains representing the first, second, and third chromosomes to 12-hour exposures to 36°.5 C. at specific periods in development.

2. End-results of temperature change during development are often similar to those produced by mutant genes when the temperature is constant. In other words, an individual of one genetic constitution raised at one temperature resembles phenotypically an individual of a different genetic constitution raised at another temperature.

3. The time of appearance of phenocopies agrees with facts already known about temperature-effective periods (TEP's) in the ontogeny of *Drosophila*.

4. The temperature-effective periods for different characters may vary in duration and may occur at different periods in development.

5. Some TEP's occur before the morphological ontogenetic processes affected.

6. Other TEP's are specific for the morphological developmental period of the organ concerned.

7. A number of cases of reversal of dominance in varying degrees are presented.

8. The degree of dominance of a gene is a function of the environment within which the developmental processes take place.

9. The production of phenocopies and the reversal of dominance are considered in the light of the current rate theory of development.

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SOMATIC EFFECTS OF TEMPERATURE ON DEVELOPMENT IN DROSOPHILA MELANOGASTER. II. TEMPERATURE-EFFECTIVE PERIOD OF TRUNCATE

(One figure)

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PHENOTYPIC reversal of dominance in *Drosophila* as a result of temperature treatment was first reported by Goldschmidt (1935) for the genes ebony and vestigial. Since then, Child, Blanc, and Plough (1940) have demonstrated that the phenotypic expression of many nominally recessive genotypes could be induced in heterozygotes of *Drosophila melanogaster* by means of a 12-hour exposure to 36°.5 C. at fairly definite periods during larval and pupal development. They considered that these sensitive periods represented developmental or physiological stages in growth during which the rates of certain "gene-controlled reactions" could be influenced by adequate variation of the temperature environment. Two of the authors have conducted further experiments in an effort to determine accurately the temperature-effective periods (hereafter called "TEP") for one or more characters. Puparium formation was chosen as a definitive stage in developmental physiology, and the pupae were subjected to heat treatments during the 36-hour period immediately following this point in development. Special study has been made of the phenotypes truncate wing and vortex, both of which are phenotypic expressions of the genotype dumpy (dp).

MATERIALS AND METHODS

The incubators used were the two-shelf Bridges type, maintained at temperatures which were constant at any one point within ±0°.2 C. They were placed in a cold room in which the temperature was held at 15° ± 2° C. at a relative humidity of 65 ± 5 per cent. This equipment has been fully described in *Drosophila Information Service*, Bulletin 6. A wild stock (Florida +10), inbred for 1 year by sib-matings and selected for uniformity, was used in these experiments. This was crossed with a 2ple stock which had not been inbred. The control temperature for the first few experiments was held at 25° C. The high temperature used for heating was 36°.5 C., to which larvae and pupae were subjected for a period of 12 hours beginning at 5–5½ days after egg-laying.

Egg-laying periods were, for the most part, 2–3 hours in length. A shorter period cut down considerably the number of eggs obtainable. Best results were obtained by allowing females to lay and larvae to develop in bottles. The bottles retained heat longer than did vials when removed from the incubator. Yeast was added from time to time to keep the supply in the bottles plentiful. Child (1935*a*) has observed that under these conditions no effects of crowding can be observed with as many as three hundred larvae per half-pint bottle, while, on the other hand, a similar environment and a more homogeneous population result from more individuals in fewer cultures. However, bottles should not be used for heating experiments, since vials, as already noted, react more quickly to changes in temperature. Various methods were used to collect and count eggs. Best results were obtained by keeping mass matings of 4–7-day-old flies in half-pint bottles inverted over milk-bottle caps on which fresh food had been smeared. A thin, flexible scalpel was used to transfer the pupae from the culture bottles to 2 per cent agar slants. Transfers were made every 2–4 hours.

EXPERIMENTAL

The fact that truncate and vortex—pleiotropic effects of the gene dumpy—are practically unique in heterozygous 2ple stock has already been described (Child, Blanc, and Plough, 1940) as a case of reversal of dominance. The TEP for this phenomenon was localized between 4.5 and 5.5 days, or just after the time of puparium formation. The accuracy of this determination, however, was conditioned by two observations. (1) Variability in extent of effect was noticed, suggesting that individual flies were receiving different amounts of treatment. This might mean that these individuals were subjected to heat treatment during different portions of the TEP. (2) The data were tabulated according to total age of flies at time of treatment. This introduced an element of error in timing, for it was noted that egg-larval age of flies developing at 25° C. from eggs laid in a 2–3-hour period varied as much as 24 hours in length.

TABLE 1

RELATIONSHIP BETWEEN TEP FOR TRUNCATE AND TIME OF PUPATION: MALES

(Ratio of flies showing truncate effect to total number of flies in group)

TOTAL AGE IN HOURS	NUMBER OF HOURS SPENT AS PUPAE BEFORE HEAT TREATMENT																	TOTAL NUMBER OF FLIES
	6–9	6–12	7–12	8–10	9–12	10–12	11–15	12–14	12–15	12–15½	14–16	15–18	15–19	16–18	16–20	18–20	20–22	19–23	
118–22				0:4		0:10		12:12			10:12			0:4		0:2			22:44
116–26			2:3				12:13						0:15					0:16	14:47
120–24				0:4		2:17		11:11			12:21			0:4		0:2	0:1		25:61
120–26	1:18				0:18				39:52*			0:14							40:102
122–26								1:1			11:15			0:7		0:7	0:4		12:34
121–29					0:8					19:19					11:16		0:6		30:49
119–33		4:15					9:12												13:27
Total number of flies	1:18	4:15	2:3	0:8	0:26	2:27	21:25	24:24	39:52*	19:19	33:48	0:14	0:15	0:15	11:16	0:11	0:11	0:16	

* Pointed wings not included in this group.

It may be suggested then that flies of the same chronological age differ in developmental or physiological age. Since the TEP for truncate occurs in early pupal life, it was deemed likely that calculation of the TEP in terms of pupal age would reduce to a minimum the variation introduced by differences of physiological age in a population. To this end, a series was run in which an accurate check was kept of both total age and pupal age at time of treatment.

Tables 1 and 2 show the relationship between the TEP for truncate and time of pupation. The age of the flies at the beginning of the period of heat treatment is represented in hours; total age from time of egg-laying is represented on the vertical axis; and pupal age, or age from time of puparium formation, is indicated on the horizontal axis. Each fraction represents the ratio of flies showing the truncate effect to total number of flies in the group.

The data show that the TEP for truncate is more closely related to pupal age than to total chronological age, i.e., age from time of egg-laying. Truncates are found throughout when the data are tabulated on the basis of total age, whereas the period of effect is largely localized at 12–16 hours of pupal age.

This type of correspondence is to be expected. As Child (1935*b*) and Margolis (1935*b*) have observed, a TEP for a character in a population is the sum of two quantities: the TEP's in the individuals, and the variation from fly to fly in the time of occurrence of their TEP's. Thus, flies developing at 25° may reach the period of puparium formation within a time range of 4½–5½ days (Tables 1 and 2). This spread in time of development has been noted by many investigators and has been carefully and extensively studied by Powsner (1935). He attempted to reduce this variability by extreme control of temperature, food, and other environmental conditions and by using a stock inbred and selected for 3 years. The residual variability was still present. Under these conditions, flies of the same chronological age may differ in physiological age; and, conversely, flies of the same pupal age may differ as much as 24 hours in actual age. Pupation is a physiological landmark in the development of the organism. A TEP is a specific time in the developmental physiology of the organism during which certain thermolabile developmental processes occur. A much closer relation holds, therefore, between TEP and pupal age than between TEP and total chronological age.

TABLE 2

RELATIONSHIP BETWEEN TEP FOR TRUNCATE AND TIME OF PUPATION: FEMALES

(Ratio of flies showing truncate effect to total number of flies in group)

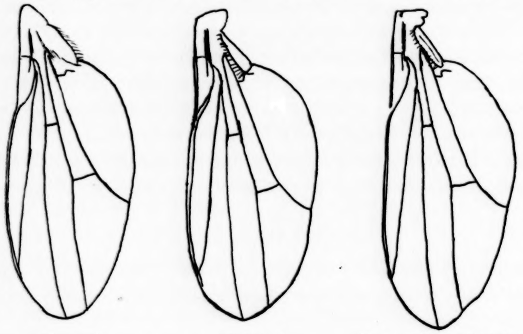
TOTAL AGE IN HOURS	NUMBER OF HOURS SPENT AS PUPAE BEFORE HEAT TREATMENT																		TOTAL NUMBER OF FLIES
	4–8	6–12	7–11	8–10	9–12	10–12	11–15	12–14	12–15	12–15½	14–16	15–18	15–19	16–18	16–20	18–20	20–22	19–23	
118–22	....	....	....	0:10	....	1:11	....	14:16	....	....	9:11	....	....	....	....	....	....	....	24:38
116–26	0:7	2:6	....	....	....	....	10:19	....	....	....	....	....	0:17	....	....	....	....	0:11	12:60
120–24	....	....	....	1:6	....	2:14	....	15:16	....	....	2:7	....	....	0:6	....	....	0:1	....	20:50
120–26	....	....	....	....	1:44	....	....	....	16:40*	....	....	0:13	....	....	....	....	....	....	19:97
122–26	....	....	....	....	....	0:1	....	1:3	....	....	1:11	....	....	0:6	....	0:7	0:3	....	2:31
121–29	....	....	....	....	....	....	....	....	....	27:30	....	....	....	....	1:11	....	....	....	28:41
119–33	....	2:20	1:9	....	....	....	10:12	....	....	....	....	....	....	....	....	....	....	....	13:41
Total number of flies....	0:7	4:26	1:9	1:16	1:44	3:26	20:31	30:35	18:40*	27:30	12:29	0:13	0:17	0:12	1:11	0:7	0:4	0:11	....

* Pointed wings not included in this group.

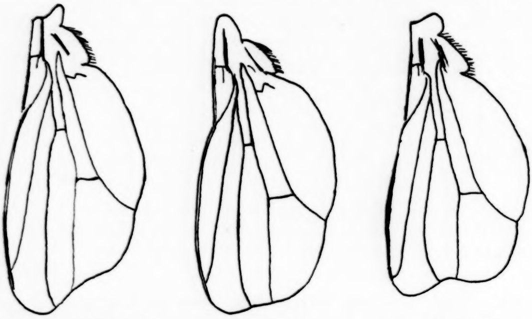
TRUNCATE

The data may therefore be tabulated according to pupal age at time of treatment. This has been done in Table 3. The truncate effect is variable; different grades of wing shape may be found, varying from nearly normal through a simulation of the phenotype of the oblique allele to a dumpy as extreme as the wings of homozygous dumpy flies raised at 25° C. In this table the truncates have been separated into three groups, representing three degrees of effect—pointed, truncate and pointed, and truncate (Fig. 1).

Pointed wing, with the same TEP as truncate, seems to be an intermediate step between truncate and normal wing. The inner distal third of the wing is flattened obliquely. It is a variable character, merging into normal and showing gradations toward slight truncate. That it is a partial truncate is further borne out in that it appears where a less marked truncate is expected—e.g., in females, in heat-treated stocks not carrying dumpy, and in stocks heated less than 12 hours.



Normal Pointed Truncate and Pointed



Truncate and Pointed Truncate Truncate

FIG. 1.—Normal and certain mutant wings in *Drosophila*

TRUNCATE AND POINTED

The third category in Table 3 includes those flies in which both the truncate and pointed patterns could be discerned.

The results included in Table 3 show that the TEP for truncate begins at 12–16 hours of pupal life. Some truncates have been obtained in earlier periods, while the sensitive period for one group of flies occurs a few hours later. But the body of evidence substantiates the 12–16-hour TEP.

Whether or not the TEP is identical for males and females cannot be ascertained from the data. A difference in degree of effect, however, is apparent. The truncate effect is qualitatively and quantitatively greater in males than in females. The percentage of occurrence of truncate and pointed is 100 per cent for males and 86 per cent for females in the 12–14-hour period, and 68.8 per cent for males and 41.4 per cent for females in the 14–16-hour period. Considering the two phenotypes separately, in the 12–14-hour period 100 per cent of the males show truncate and 8.3 per cent of them show pointed, while 68.6 per cent of the females show truncate and 28.6 per cent show pointed. In the 14–16-hour period 64.6 per cent of the males show truncate and 27.1 per cent show pointed, while 27.6 per cent of the females show truncate and 20.7 per cent show

TABLE 3

DEGREE OF MANIFESTATION OF TRUNCATE

(Relation between age as pupae at time of treatment and number of pupae affected)

PUPAL AGE BEFORE HEAT TREATMENT (IN HOURS)	FEMALES				MALES			
	Truncate	Truncate and Pointed	Pointed	Number of Pupae Treated	Truncate	Truncate and Pointed	Pointed	Number of Pupae Treated
6–10	....	....	1	7	....	....	....	3
7–11	1	....	1	13	5	....	....	13
8–10	1	....	....	16	....	....	....	9
9–12	....	....	....	8	....	....	....	12
10–12	3	....	....	26	2	....	....	27
11–15	11	....	8	30	18	1	2	25
12–14	20	4	6	35	22	2	....	24
12–16	22	4	2	31	17	1	1	19
14–16	6	2	4	29	20	11	2	48
15–19	....	....	....	21	....	....	1	19
16–18	....	....	....	12	....	....	....	15
16–20	....	1	....	11	10	....	1	16
18–20	....	....	....	7	....	....	....	11
19–23	....	....	....	12	....	....	....	16
20–22	....	....	....	5	....	....	....	11

TABLE 4

GRADES OF TRUNCATE FOLLOWING TREATMENT AT DIFFERENT AGES

(In percentage)

GRADES OF TRUNCATE IN DESCENDING ORDER OF MAGNITUDE	PUPAL AGE AT START OF TREATMENT			
	12–14 Hours		14–16 Hours	
	Females	Males	Females	Males
Truncate (maximum)	66.7	91.7	50.0	60.6
Truncate and pointed (intermediate)	13.3	8.3	16.7	33.3
Pointed (minimum)	20.0	0.0	33.3	6.1
Total percentage	100.0	100.0	100.0	100.0

pointed. Equating at 100 per cent the total number of flies showing an effect, the relationships are as shown in Table 4. With pointed considered as a minimal grade of truncate and with flies showing both pointed and slight truncate as intermediates, it is clear that the truncate effect is more pronounced, i.e., qualitatively greater, in males than in females.

VORTEX

Vortex, another phenotypic expression of dp, also appeared as a result of heat treatment of heterozygous 2ple stock. Its TEP is earlier than that of truncate, i.e., beginning at approximately 8–10 hours of pupal life. The data are summarized in Table 5. The phenotype varied from slight whorling of microchaetes to distinct humps on the thorax. Vortex also appeared in the treated heterozygous crossveinless (cvc in chromosome 3).

The appearance of vortex in a group of treated flies was associated with the occurrence of a number of undeveloped or abnormal pupae. These were similar to the series described by Plough and Child (1936). They included flies with only the thorax, with head in thorax, etc., as well as a number of "white" pupae, in which nothing but a white viscous liquid was found surrounding a number of unhistolyzed larvae organs.

Vortex also appeared in a series in which the pupae were kept at 18 °C. from the time of pupation to the time of heating. (Otherwise, 25° C. was used as the control temperature.) On the basis of Powsner's (1935) figures for length of pupal period at these two temperatures, 1 hour at 25° C. equals approximately 2 hours at 18° C. It was found that vortex appeared at ages comparable to expectation on the basis of the results obtained at 25° C., viz., at 14–20 hours.

TABLE 5

TEP OF VORTEX

(Relation between age as pupae at time of treatment and number of pupae affected)

Pupal Age in Hours	Females		Males	
	Vortex	Number of Pupae Treated	Vortex	Number of Pupae Treated
0– 4	0	2	0	4
4– 8	3	3	0	2
6– 9	1	6	1	10
6–10	0	4	0	1
7–11	0	12	0	11
8–10	4	16	3	9
8–12	2	51	2	20
9–12	1	8	0	12
10–12	0	26	0	27

Most of the data discussed were obtained with the use of a 12-hour heating period. A heating period of 15 hours gave similar results, which have been included with the foregoing. Heating periods as brief as 6 hours were tried. Truncate was induced with a treatment of 9 hours, while 6 hours sufficed for manifestation of slightly pointed wing. Treatments of less than 12 hours showed the same TEP at 12–16 hours of pupal life.

Wild-type males, heterozygous 1ple females, and heterozygous cvc males and females were also treated in these experiments. Pointed wing appeared in all these stocks at 12–16 hours as a result of periods of heat as brief as 9 hours. Vortex appeared in heterozygous cvc as a result of a heat period of 12 hours.

Other phenotypes were observed to have TEP's beginning in the first 26 hours of pupal life. They were: partial crossveinless, missing microchaetes, plexus, and certain bristle effects, including forked, singed, bent, and bifurcate. In some cases microchaetes were almost completely removed. Plexus affected different cells of the wing, depending on the time of heat treatment. All these effects were specific in point of time. Induced as a result of heat treatment of 12 hours in duration, they showed sensitive periods which began within limits of 4 hours. These data will be elaborated elsewhere.

THE TRUNCATE REACTION

Certain indications are forthcoming as to the nature of the process leading to the expression of heterozygous dumpy. Auerbach (1936) has described in general terms the development of the dumpy wing. From the eversion of the wing pouch at approximately 6 hours after puparium formation to about 17–20 hours of pupal age, the wing seems to develop normally. It presents at the latter time an oblique flattening of the inner distal end of the wing, which is similar in appearance to the slight pointed effect of truncate. From this time on, the wing is subject to a progressive truncation, until it assumes its characteristic shape completely at about 35 hours of pupal life. According to Goldschmidt (1937), this effect is the result of degeneration and resorbtion of the subepithelial tissue toward the margin of the wing.

We may draw two inferences from these embryological observations: (1) the last step in the series of processes leading to truncated wing is a visible degeneration of already formed wing material (Goldschmidt, 1937); (2) there is a close temporal relationship between this degenerative process and the truncate reaction. In fact, the TEP for truncate overlaps the visible ontogenetic process of wing truncation. The possibility that both reactions are identical may not be overlooked, but for the present it remains in the field of speculation.

Further indications concerning the nature of the truncate reaction are furnished by the phenogenetic evidence. On the basis of the data presented in this paper it may be stated that heat treatment must begin 12–16 hours after puparium formation in order to produce the truncate effect. When begun at this time, heating periods of 9, 12, and 15 hours gave positive results. On the other hand, when begun in the 9–12-hour period, 12- and 15-hour treatments produced very few truncates, which probably belonged to the latter part of the period.

The type of explanation advanced by Margolis (1935*a*, 1935*b*), and later simplified (Margolis and Robertson, 1937) to describe the process of reduction of facet number, would serve equally well as a model for the truncate reaction. According to this scheme, two wing reactions (at least) are involved: (1) a wing-forming process and (2) a truncate-producing process. These are present in the normal, as well as the mutant, fly. When the first reaction exceeds the second, normal wing ensues. When the second outdoes the first, truncate results.

But the size and shape of the eventual wing depends not only on the relative rates of these reactions but also on the length of the time interval in which the latter take place. In other words, the process of general development, i.e., development of the organism as a whole, sets time limits. Therefore, the temperature treatment which produces truncate may act by affecting either or both of two processes. It may incresae (or decrease) the rate of the truncate-producing reaction, or it may increase (or decrease) the effective time of reaction.

In terms of the rate theory of development the wing reactions presumably have differing temperature coefficients. When the dumpy gene is present, the temperature coefficient of the truncate-producing reaction is higher than that of the building-up process, so that, as a result of temperature treatment, the degeneration-rate of the reaction may surpass the rate of the building-up process. The result would be more wing substance (or precursor thereof) destroyed than formed during the TEP. It should be borne in mind that the nature of the result may be determined by the relative value of the temperature coefficient for development as a whole. Other mechanisms may also apply. If actual degeneration of wing tissue is not involved, another explanation must be sought.

SUMMARY

1. Study has been made of the characters truncate and vortex, phenotypic expressions of the second chromosomal gene dumpy, which were manifested in heterozygous 2ple stock as a result of high temperature treatment subsequent to the time of puparium formation.
2. The temperature-effective period (TEP) for truncate is more closely related to pupal age than to total chronological age.
3. The TEP for truncate begins at 12–16 hours of pupal life.
4. Truncate wings present a continuous variation from normal to extreme dumpy.
5. The truncate effect is quantitatively and qualitatively greater in males than in females.
6. The TEP of vortex begins at approximately 8–10 hours of pupal life.
7. Heating periods of 12 and 15 hours give similar results, while shorter periods give less pronounced results.
8. Stocks other than heterozygous 2ple showed a slight truncate effect as a result of heat treatment during the same 12–16-hour period, while vortex appeared in one of these stocks.
9. Other phenotypes—partial crossveinless, missing microchaetes, plexus, and certain bristle effects—were observed to have TEP's beginning in the first 26 hours of pupal life.
10. The nature of the truncate reaction was discussed on the basis of the known embryological and phenogenetic facts, as interpreted from the point of view of the rate theory of development.

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THE HEAT PRODUCTION AND OXYGEN CONSUMPTION OF PUPAE OF GALLERIA MELLONELLA AT DIFFERENT CONSTANT TEMPERATURES

(One figure)

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IN A number of insects the rate of respiratory metabolism is not constant throughout the pupal period but changes in a characteristic manner. The relatively high rate early in the pupal life decreases to a minimum and then rises, giving a U-shaped curve for the respiratory metabolism during this period. This type of curve for oxygen consumption and for carbon dioxide production has been reported by numerous investigators. Taylor (1927), Taylor and Steinbach (1931), and Crescitelli (1935), in connection with such curves, have noted a terminal drop in metabolic rate just before emergence in *Galleria mellonella*. Krogh (1914) and Janda and Kocián (1933) made studies concerning the effect of temperature on the metabolism of *Tenebrio molitor* pupae, and they conclude that in this species the effect of increasing temperatures is to shorten the duration of the pupal period and to raise the general level of the curves. Krogh's results indicate also that the total amount of carbon dioxide produced during the pupal period of *Tenebrio* is the same at all temperatures studied. Crescitelli (1935) investigated the effect of certain temperatures between 20° and 39°.97 C., inclusive, on the respiratory metabolism of *G. mellonella* pupae; he also found that increasing temperatures tend to elevate the level of the metabolism curves and, within certain limits, to shorten the length of the pupal period. However, his results indicate that the total amounts of oxygen consumed and of carbon dioxide produced are not the same at all temperatures but that there is an optimum temperature (30° C.) at which development is accomplished with a minimum total gaseous exchange.

The heat production of insect pupae has been less extensively studied. Bialaszewicz (1933) found that in *Lymantria dispar* the rate of heat production during the pupal period deceased to a minimum during the first half of the period and then increased, giving a U-shaped curve for the rate of heat production throughout pupal life. Balzam (1933) reported rates of heat production and oxygen consumption during the metamorphosis of *L. dispar.* He obtained U-shaped curves from both types of measurements and found an approximate parallelism between the two. Taylor and Crescitelli (1937), working with *G. mellonella,* have obtained rate curves for heat production which approximately parallel those for oxygen consumption during metamorphosis, both types being of a U-shape, followed by a marked drop near the time of emergence.

There seems, however, to be no literature concerning the effects of temperature on heat production during insect metamorphosis. Consequently, it seemed advisable to make such a study in conjunction with a study of the respiratory metabolism of *G. mellonella* during metamorphosis, and to relate it, if possible, to the measurements of heat production and oxygen consumption of this animal at 30° C., reported by Taylor and Crescitelli (1937).[1]

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[1] This paper was presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Brown University. The writer wishes to acknowledge her indebtedness to Professor Ivon R. Taylor for valuable advice and assistance.

MATERIALS AND METHODS

The bee moth, *G. mellonella*, which was used in this investigation, was obtained from the stock which has been cultured at the Brown University laboratory, and the pupation time of each of the animals employed was known to within an hour. The rates of heat production and of oxygen consumption of individual pupae, at a particular constant temperature, were measured alternately throughout the entire period from the time of pupation up to the time of emergence. The studies were made at each of the following temperatures: 25°, 35°, and 40° C., which were obtained by the use of a constant-temperature water bath arranged so as to maintain each temperature to within ± 0°015 C.

The apparatus and method described by Taylor and Crescitelli (1937) were used in making measurements of heat production of individual pupae during development. Sensitive differential manometers, calibrated by the formula method given by Dixon (1934), were employed in making the oxygen-consumption measurements. The routine procedure used in making the latter measurements was essentially similar to that described by Taylor and Steinbach (1931). As soon after pupation as possible (from 1 to 5 hours), each animal was removed from its cocoon, weighed, and the sex noted. Usually two animals were selected at the same time, and the heat-production measurements were made on one simultaneously with the oxygen-consumption measurements of the other; the animals were then alternated. Such measurements, each covering a period of 30 minutes, were made at about 8-hour intervals throughout the pupal life of the insect. Between measurements, as well as during them, the animals were maintained at the constant experimental temperature under consideration. The loss of weight of the animals was determined by weighing every 24 hours.

The procedure outlined above has been followed in the measurements of heat production and oxygen consumption of *G. mellonella* pupae at three constant temperatures: at 25° and at 35° with ten males and ten females at each temperature and at 40° using six animals of each sex.

RESULTS AND DISCUSSION

THE MEASURED RATES OF HEAT PRODUCTION AND OXYGEN CONSUMPTION

The individual and composite curves obtained from these measurements are very similar in form to those obtained at 30° C. by Taylor and Crescitelli (1937). The values of Taylor and Crescitelli's curves for heat production and oxygen consumption fall, for the most part, between those obtained here at 25° and 35°, as is to be expected. In regard to their heat-production measurements, however, Taylor and Crescitelli have pointed out that, although the pupae have chitinous cases, there is probably some loss of water through the spiracles, and that this evaporation would tend to lower the values obtained when the heat production was measured. They further suggest that, from data on the decrease in weight of the animals and the loss due to elimination of carbon dioxide, it would be possible to estimate the quantity of heat used in this evaporation. In the present work such corrections for heat-production measurements have been calculated; and in doing this, several interesting observations have been made. These will be mentioned in connection with the procedures involved in making the corrections.

CORRECTION OF HEAT-PRODUCTION MEASUREMENTS FOR THE LOSS OF HEAT OWING TO EVAPORATION OF WATER

a) *Rates of weight loss.*—Since the animals were weighed every 24 hours, it was possible to plot individual curves, and consequently composite curves representing the weight of the pupae at various stages of development. Table 1 shows the average percentage of weight lost in each sex at each of the experimental temperatures. These data indicate that the average percentage of loss in the females is slightly greater than in the males at all of the temperatures studied. More significant, perhaps, is the effect of the high temperature (40°) as seen here. An increase from 25° to 35° does not seem to alter the weight loss greatly, but an increase from 35° to 40° results in a considerably greater percentage of weight lost in both sexes.

TABLE 1

AVERAGE PERCENTAGE OF WEIGHT LOST DURING PUPAL PERIOD

Temperature (°C.)	Males	Females
25	16.1	19.8
35	15.0	18.9
40	23.7	26.5

The composite weight curves were converted into curves showing the rates of weight loss throughout metamorphosis, in order to apply them in the correction of the heat-production rate curves.

b) *Rates of weight gain owing to respiration.*—Aside from the evaporation of water, probably the only other factor involved in change in weight of the pupae is respiration. If the respiratory quotient of the animal were one, the volumes of oxygen consumed and carbon dioxide given off would be the same, and the weight change would be equivalent to the difference in density of the two gases multiplied by the volume of oxygen consumed. Thus, if the R.Q. were one and the volume of oxygen consumed per unit of time were a liter,

$$\text{R.Q.} = \frac{\text{Vol. CO}_2\text{ evolved per unit of time}}{\text{Vol. O}_2\text{ consumed per unit of time}} = \frac{1\text{ liter}}{1\text{ liter}}\,.$$

On the basis of the density of the two gases at 25° C., this ratio at that temperature becomes 1.81105 gm./1.30915 gm. In this case the change in weight on account of respiration would be equal to −0.5019 gm. and would indicate a loss of 0.5019 gm. for the consumption on 1 liter of oxygen. But the work of Taylor and Steinbach (1931) indicates that throughout the pupal period of *Galleria* at 30° C. the R.Q. remains constant at 0.69, and Crescitelli (1935) has shown this value to be independent of temperature. When the foregoing ratio is modified for the R.Q. of the animal, we have the ratio equal to 1.24962 gm./1.30915 gm. at 25° C., and the difference in weight because of the consumption of 1 liter of oxygen would be +0.05943 gm. Thus the animal actually gains in weight, owing to respiration. By reducing this value for weight gain to milligrams and by dividing by the weight (in milligrams) of a liter of oxygen at 25°, the factor representing the change (gain) in weight of the animal because of the consumption of 1 mg.

of oxygen at 25° was obtained. In a similar manner the factor for each of the other temperatures was calculated. By means of these factors the rates of gain of weight have been calculated for the animals at each of the three temperatures.

c) *Rates of evaporation of water*.—The observed change in weight of the animals represents the loss of weight by water evaporation plus the gain in weight owing to respiration. Hence, the actual rate of loss of water from the animals is the sum of the observed rate of weight loss and the calculated rate of weight gain, owing to the respiratory exchange mentioned above. Curves showing the actual rates of water loss throughout metamorphosis for each sex at each temperature were plotted, and the area under each was measured with a planimeter in order to determine the average total weight of water lost per gram of animal. Table 2 shows the results of these measurements. From these data it appears that an increase in temperature from 25° to 35° only slightly alters the average total weight of water lost. However, an increase from 35° to 40° results in a considerably greater loss. During these experiments none of the forty animals kept at 25° and 35° failed to emerge. On the other hand, out of the eighteen selected for measurements at 40°, only twelve emerged, although in most cases the development seemed to progress normally up to the time of emergence. Crescitelli (1935) showed that a temperature of 39°.97 C. resulted in the retardation of the developmental processes in this insect, but he has not attempted to explain the nature of the deleterious effects produced by the high temperature. In the light of the data shown in Table 2 it seems that, among other things, the high temperature might result in excessive drying and hardening of the pupal coverings. Thus, even should the animals develop normally, they might not be able to escape from the hardened cases inclosing them.

TABLE 2

AVERAGE TOTAL WEIGHT OF WATER LOST PER GRAM OF ANIMAL

Temperature (° C.)	Males (Gm.)	Females (Gm.)
25	0.176	0.221
35	0.161	0.210
40	0.261	0.309

TABLE 3

AVERAGE TOTAL AMOUNTS OF HEAT (GRAM CALORIES PER GRAM) LOST BY EVAPORATION OF WATER

Temperature (° C.)	Males	Females
25	102.30	128.90
35	92.58	121.08
40	150.00	177.17

d) *Rates of heat loss because of the evaporation of water*.—The rates of heat loss because of evaporation were calculated from the rates of water loss throughout metamorphosis, and the results were plotted. By measuring the area under these curves the average total amounts of heat lost by the evaporation of water were obtained, and they are presented in Table 3.

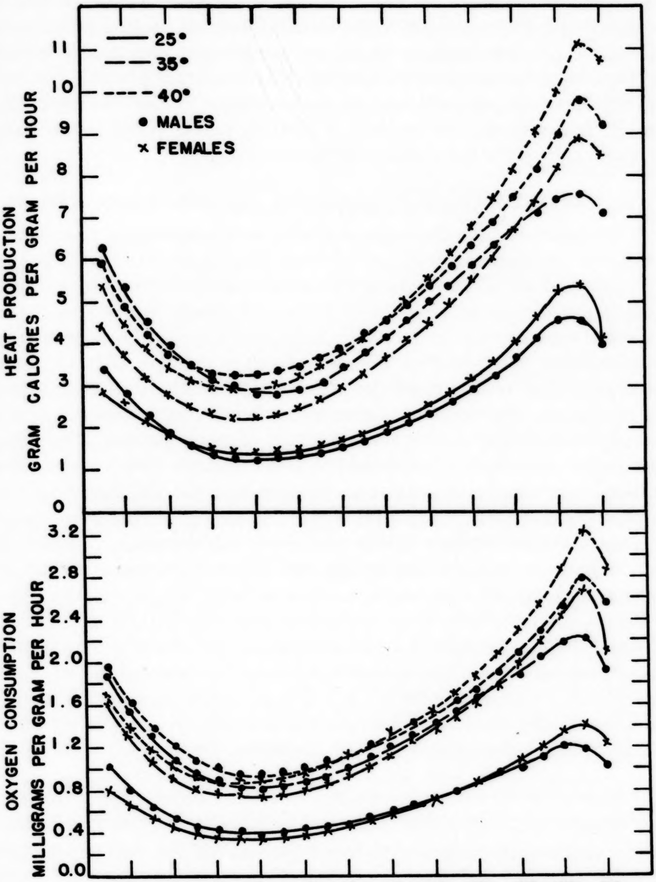

FIG. 1.—Composite curves showing rates of heat production and oxygen consumption for male and female pupae at 25°, 35°, and 40° C.

When the values of the calculated rates of heat loss were added to those of the rates of heat production as measured in these experiments, the values shown in Figure 1 were obtained. In this figure are shown composite curves representing the corrected rates of heat production in gram calories per gram of animal per hour, and the oxygen consumption in milligrams per gram of animal per hour, for each of the sexes at 25°, 35°, and 40° C. Because of the variation in length of pupal life, even in animals of the same sex at the same temperature, it was considered advisable not to plot the composite curves against age in hours but rather to let the total abscissa represent the period of pupal life and the intervals of this abscissa to represent fractions of this period. For convenience, each interval has been arbitrarily chosen to represent one twenty-fourth of the time (in hours) necessary for pupal development. This makes it possible for each composite curve to represent, at all points, comparable stages in the development of the individual animals. In addition, this method of plotting makes possible the direct comparison of composite curves for the different temperatures.

EFFECT OF TEMPERATURE ON HEAT PRODUCTION AND OXYGEN CONSUMPTION

In Figure 1 it is apparent that the typical U-shaped curve with a pronounced drop just before emergence represents the rates of both heat production and oxygen consumption at 25°, 35°, and 40° C. throughout the development of the pupae. Taylor and Crescitelli (1937) found this to be true at 30° C.; and Balzam (1933), working with *L. dispar* pupae at room temperature, reported a U-shaped curve for both heat production and oxygen consumption. The data in Figure 1 further indicate that an increase in temperature from 25° to 35° results in an elevation of the metabolic rates at all stages of development of the pupae. The amount of elevation of each stage, however, does not appear to be constant, being greater during the latter part of metamorphosis than during the early and middle parts. Although it is believed by most workers that both histolytic and histogenic processes occur simultaneously throughout pupal life, the histolytic processes are thought to predominate early in the period and the histogenic processes to predominate in the latter part. Assuming that this is true, there would seem to be some differential effect of this increase in temperature on the two types of processes, as indicated by the heat production and oxygen consumption. The increase in temperature from 35° to 40° apparently has a peculiar effect. During the first part of pupal life at 40° the curves have about the same values as those for the animals at 35°. Soon after the middle of the period, however, an increase in the level of the former becomes apparent, and by the end of development this increase is marked. In the light of the assumption made above, it would appear that this increase in temperature has relatively little effect on the histolytic processes but that it does accelerate the histogenic processes.

EFFECT OF TEMPERATURE ON THE AVERAGE TOTAL HEAT PRODUCTION AND THE AVERAGE TOTAL OXYGEN CONSUMPTION DURING PUPAL DEVELOPMENT

The average total amount of heat produced (including the correction for water evaporation) and the average total amount of oxygen consumed during the entire period have been calculated from the composite curves (Fig. 1) by measuring the area under each. With the data shown in Table 3 the percentage of the corrected total heat production which was used in the evaporation of water was calculated. The results of these calculations are shown in Table 4. In the work of Taylor and Crescitelli (1937) the average total amount of heat produced and the average total amount of oxygen consumed at 30° have not been calculated. However, if it can be assumed that the average total loss of heat due to evaporation of water at 30° is about the same as the average for the three temperatures used here (15.3 per cent for the males and 17.8 per cent for the females), the average total heat production at 30° can be estimated from their curves.

For purposes of comparison this assumption has been made, and the average total heat production at that temperature has been calculated upon this basis and is included in Table 5. Crescitelli (1935) has calculated the average total amounts of oxygen consumed at a number of temperatures, one being 30°05 C., which is practically identical with that (30° C.) used by Taylor and Crescitelli (1937). Table 5 shows a comparison of the data obtained in these three separate investigations.

From these combined data it appears that neither the average total amount of heat produced nor the average total amount of oxygen consumed is constant over temperatures ranging from 25° to 40° C. Crescitelli concluded that neither the average total oxygen consumption nor the average total carbon dioxide production was constant over a range of temperatures from 20° to 39°97 C., inclusive, but that there was a minimum for each at 30°05. There is good agreement between the results of the present work and that of Crescitelli concerning the average total oxygen consumption at 25°, 35°, and 40°.

TABLE 4

PERCENTAGE OF TOTAL CORRECTED HEAT LOST OWING TO EVAPORATION OF WATER

Temperature (° C.)	Males	Females
25	16.3	18.5
35	13.9	18.1
40	15.6	16.8
Av.	15.3	17.8

TABLE 5

AVERAGE TOTAL AMOUNTS OF HEAT PRODUCED AND AVERAGE TOTAL AMOUNTS OF OXYGEN CONSUMED DURING PUPAL DEVELOPMENT

TEMPERATURE (° C.)	HEAT (GRAM CALORIES PER GRAM)		O_2 (MILLIGRAMS PER GRAM)	
	Males	Females	Males	Females
25	625.78	698.04	180.72	191.91
30	522.10	629.00	175.84	196.99
35	668.44	667.35	195.88	208.67
40	960.50	1,057.86	282.10	313.62

THE CALORIFIC QUOTIENT

The calorific quotient is the number expressing the amount of heat evolved in gram calories per milligram of oxygen consumed. Zuntz, Pfluger, and Rubner (quoted by Needham, 1931) have found this number to be 3.2 when protein is burned, 3.3 for combustion of fat, and 3.5 for carbohydrate. The value of this ratio, like that of the respiratory quotient, is supposed to vary according to the particular type of foodstuff undergoing metabolism. The differences between these numbers, however, are very small; and therefore it is difficult to attach a great deal of significance to calorific quotients obtained in animals experimentally. Nevertheless, in connection with the present work the calorific quotients at various stages of development have been calculated and are presented in Table 6. It will be noted that there is some variation in these values during metamorphosis. Balzam (1933) reported that during metamorphosis of *L. dispar* the calorific quotient values give a V-shaped curve. They diminish at first to a minimum (2.3) and then later mount to the initial level (3.2) of metamorphosis, the minimum falling toward the middle of the period. However, it appears to the writer that the variation obtained in the present work is neither great enough nor consistent enough to warrant such a conclusion in *G. mellonella* at any of the temperatures employed.

TABLE 6

CALORIFIC QUOTIENT FOR VARIOUS STAGES OF DEVELOPMENT AT 25°, 35°, AND 40° C.

INTERVALS (FRACTIONS OF DEVELOPMENTAL TIME, IN TWENTY-FOURTHS)	25°		35°		40°	
	Males	Females	Males	Females	Males	Females
1	3.31	3.52	3.19	2.66	3.01	3.07
2	3.49	3.97	3.24	2.73	3.04	3.18
3	3.52	3.88	3.34	2.88	3.06	3.30
4	3.46	3.96	3.45	3.02	3.11	3.22
5	3.40	3.91	3.58	2.99	3.17	3.26
6	3.39	3.87	3.57	2.98	3.23	3.22
7	3.28	3.82	3.58	2.89	3.34	3.17
8	3.34	3.77	3.42	2.87	3.42	3.17
9	3.21	3.78	3.35	2.91	3.43	3.90
10	3.25	3.78	3.38	2.93	3.45	3.27
11	3.19	3.60	3.39	3.00	3.44	3.25
12	3.22	3.53	3.42	3.05	3.46	3.32
13	3.10	3.38	3.41	3.13	3.51	3.36
14	3.14	3.20	3.47	3.21	3.47	3.40
15	3.14	3.37	3.47	3.17	3.50	3.52
16	3.16	3.49	3.45	3.23	3.53	3.54
17	3.25	3.54	3.51	3.29	3.56	3.57
18	3.29	3.68	3.52	3.33	3.59	3.54
19	3.44	3.79	3.51	3.35	3.61	3.55
20	3.57	3.79	3.52	3.40	3.58	3.50
21	3.65	3.90	3.45	3.37	3.54	3.52
22	3.73	3.83	3.39	3.36	3.55	3.45
23	3.84	3.84	3.37	3.32	3.51	3.43
24	3.86	3.25	3.65	3.98	3.60	3.70
Av.	3.38	3.69	3.43	3.13	3.40	3.39

In Table 6 the values for the calorific quotient are considerably higher than those obtained by Taylor and Crescitelli (1937) for *Galleria* pupae at 30°. However, as has been noted earlier, in the work of those investigators the heat loss due to the evaporation of water has not been calculated, as was done in this work; and it seems probable that the values obtained there would be higher if this were done. In connection with the low values which he obtained in *L. dispar*, Balzam suggests that endothermic reactions occurring during metamorphosis might be responsible. However, he makes no mention of the possibility of heat loss due to evaporation of water, and this might also be a factor causing the low calorific quotients which he obtained.

SUMMARY AND CONCLUSIONS

1. Measurements of the heat production and of the oxygen consumption have been made at 25°, 35°, and 40° C. on individual *G. mellonella* pupae throughout development.

2. The measured rates of heat production have been corrected for the heat used in the evaporation of water from the animals. In making this correction the following observations have been made. (*a*) An increase in temperature from 25° to 35° does not appreciably change the average percentage of weight lost in the pupae, but an increase from 35° to 40° results in a considerably greater loss in both sexes. (*b*) The change in weight due to respiration is an actual gain, and the rates of such gains at the three temperatures under investigation have been calculated. (*c*) The rates of loss of water from the pupae are not greatly altered by an increase in temperature from 25° to 35°, but an increase from 35° to 40° results in a much greater loss of water.

3. An increase in temperature from 25° to 35° results in an acceleration of the metabolic processes at all stages of development, but this effect is greatest during the latter part of the period. An increase in temperature from 35° to 40° does not appreciably alter the rate of metabolism during the early part of metamorphosis, but during the latter part of the period the rate is greatly increased.

4. The average total amount of heat produced by the animals at 35° is approximately the same as that produced by animals at 25°; the same was found to be true in the case of the oxygen consumption. In animals kept at 40°, however, there is a much greater average total heat production and oxygen consumption than at either of the other two temperatures.

5. The calorific quotients for various stages of development have been obtained. The average calorific quotients for the pupal period vary from 3.13 to 3.69.

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TEMPERATURE CHARACTERISTICS OF THE ELECTRICAL POTENTIAL IN FROG SKIN

(Three figures)

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A PREVIOUS paper (Barnes, 1939) described the effect of large and abrupt changes of temperature on the electrical potential of frog skin. The present report deals with the effect of more gradual changes in temperature in an attempt to determine the temperature characteristic. The earlier literature is reviewed in the previous paper. It will be recalled that Lesser (1907) believed that the response of the skin to temperature indicated the physical nature of the electrical potential, while Lund and Moorman (1931) obtained a Q_{10} of 2 (based on average potential over 3-hour intervals at 16.°4 and 26.°4 C.), which supported their theory of a metabolic origin of the potential. Motokawa (1938*b*) studied the effect of temperature on the action current in frog skin.

METHODS

The skin-holder described in a previous paper (Barnes, 1939) was not used in this investigation, owing to the small volume and the possibility of unequal cooling of the salt solution and the glass core on the inside of the skin.

The skin-holder used in the experiments reported in this paper afforded exactly symmetrical conditions of pressure, volume of Ringer's solution, and oxygen on each side of the skin. It was previously found (Barnes, 1939) that tying the skin on a glass tube with the morphological outside against the glass raises the potential. In the new holder the skin was held between two disks of filter paper, 2.5 cm. in diameter, each having in the center a circular opening 1 cm. in diameter. It was found by Mr. R. J. Coe (who will publish at a later date on mechanical factors affecting the potential) that the potential was stimulated if the ring of filter paper was omitted on the side pressing on the morphological outside surface of the skin. The ends of two cylindrical glass chambers each containing 25 cc. of oxygenated Ringer's pressed against each side of the skin held in the filter paper. A round window, 1 cm. in diameter, on the end of each cylinder allowed contact of the Ringer's solution with the skin. A greased glass sleeve held the chambers in place. Silver–silver chloride electrodes (having no electrode potential) dipped into the salt solution on each side. The silver wires were introduced through side arms to prevent contact with the oxygen bubbles, which escaped by separate tubes having glass bulbs to prevent the meniscus from moving up the tubes. The holder was clamped in a water bath, and potential readings were made with a Type K potentiometer. These determinations agreed with curves obtained by switching onto a Micromax recording potentiometer.

The temperature of the bath was lowered by adding ice; but, because of the small magnitude of the average potential change with temperature, increments of 5° C. were used. The rate of temperature change was about 1° C. per minute, and a constant temperature was maintained for about half an hour to allow the skin potential to come to equilibrium. Dorsal or ventral skins were kept in Ringer's solution of pH 7.5 for varying intervals before the experiment. The weight and sex of each frog and other particulars are recorded in Table 1.

TABLE 1

TEMPERATURE CHARACTERISTICS OF SKIN POTENTIAL

Date	Previous Temperature (° C.)	Sex	Weight (Gm.)	Skin	Age of Preparation (Min.)	First Temperature (° C.)	Length of Time at First Temperature (Min.)	Temperature Range of Run (° C.)	Duration of Run (Hr.)	μ-Value (Cal.)	Potentials during Run (Mv.)	Remarks
Dec. 28..	14	Female	48	Dorsal	75	28	40	25–0	4	5,750	31 –12.5	....
Dec. 30..	13	Female	29	Ventral	35	......	....	29–5	5.2	5,060	36 –17	Rose 15–23 mv. at 5°–0°. See Fig. 3
Jan. 13..	15	Female	....	Ventral	5	27	180	20–0	3	5,360	34 –12	Fell 78–34 mv. at 27°.
Jan. 17..	13.5	Female	35.5	Dorsal	105	20	60	15–5	2.6	17,250	15 – 5	Slight fall at 20°–15°
Jan. 26..	15	Male	26.5	Ventral	20	......	....	26–0	5	11,500	22.6– 2.1	Fell 46–22.6 mv. at 26°.
Feb. 17..	15	Female	40	Dorsal	180	27 and 20	130	15–5	1.5	5,018	37.2–27.3	Rose 37–40 mv. at 27°–20°
Feb. 18..	14	Female	40	Dorsal	75	20	40	15–0	3	{5,000 10,323}	40 –17.7	Low μ above 15°
Feb. 28..	15.5	Female	37	Dorsal	25	20	75	15–5	2.5	9,571	31.5–17	Slight fall at 5°–0°.
Mar. 3..	16.5	Male	....	Dorsal	125	20	75	15–5	2.2	10,750	26.7–13	Slight fall at 5°–0°
Mar. 7..	17	Female	59	Dorsal	35	......	....	20–5	4	9,966	24 – 8	2 hr. at 20°; slight drop at 5°–0°. See Fig. 1
Mar. 9..	16	Female	40	Dorsal	80	......	....	20–10	4.3	9,775	23 –12.3	Slight drop at 10°–5°
Mar. 14..	15	Male	38.5	Dorsal	25	......	....	20–0	2.7	5,661	19 – 9	Rebound at each temperature. See Fig. 1
Apr. 13..	15	Female	42.5	Ventral	45	20	60	15–0	4	9,000	48 –21	Rose 34–48 mv. with eosin at 20°

RESULTS

In spite of the notorious variations in potential, satisfactory curves were secured after the preliminary period of potential fall. Of a total of eighteen graphs obtained, only six had to be discarded, owing to fluctuations, gradual changes in potential occurring independently of temperature, or failure of the skin to respond to temperature changes. It was found that unsatisfactory results occurred at temperatures above 20° C. Engelmann (1872) reported a fall in potential at 25° C. and above. Klopp (1924) obtained sharp falls at 38° C., but he did not study the interval 21°–38° C. The frogs for my experiments were kept in a cement tank containing damp leaves at about 15° C. and were probably "acclimatized" to this temperature. A rise in potential frequently occurred between 28° or 20° and 15° C. Also, when 0° C. was reached, the potential sometimes rose slightly or failed to fall in the interval 5°–0° C. The initial interval of inconsistent potentials at 20° or above is indicated as "first temperature" in Table 1. In most of the preparations the potential approached a constant level, as indicated in Figure 1, *A*. It was previously found (Barnes, 1939) that a fall in potential produced by rapid cooling of the skin is followed by a "rebound" potential in which about 40 per cent of the lost potential is regained within 15 minutes. These rebound potentials also occurred with gradual cooling in some skins, as shown in Figure 1, *B*.

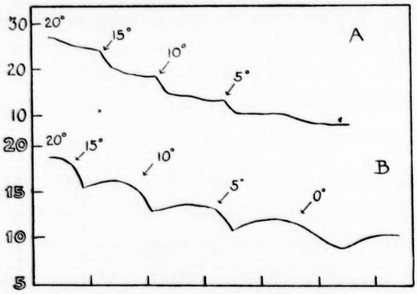

FIG. 1.—Effect of temperature on the electrical potential of frog skin. Ordinates: electromotive force in millivolts (outside surface is negative); abscissas: time in $\frac{1}{2}$-hr. intervals. *A*, dorsal skin from 59-gm. female, March 7 (see Table 1 and Fig. 2). The temperature changes are indicated at arrows. μ = 9,966 calories (calculated from last potential on "plateau"). *B*, dorsal skin from 38.5-gm. male, March 14 (see Table 1). μ = 5,661 calories calculated from low potentials before "rebound."

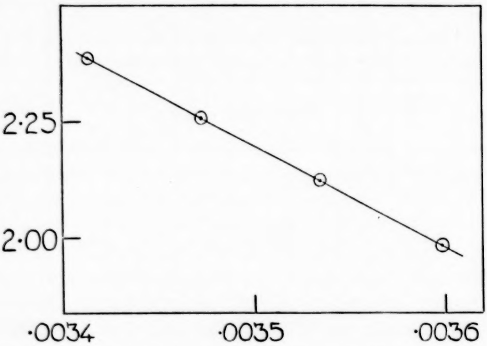

FIG. 2.—Rectilinear relation between logs of potentials (ordinates) and reciprocals of the absolute temperature (abscissas). Dorsal skin of 59-gm. female, March 7 (see Fig. 1, *A*, and Table 1). μ = 9,966 calories.

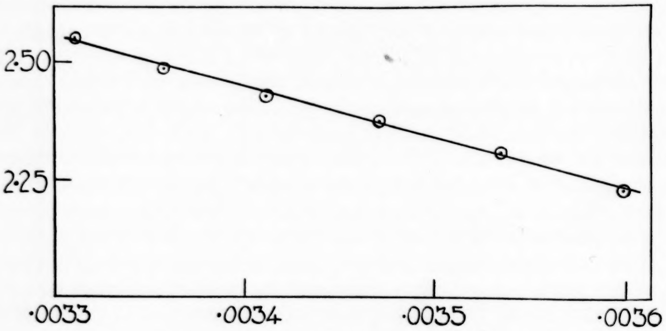

FIG. 3.—Rectilinear relation between logs of potentials and reciprocals of absolute temperature in ventral skin of 29-gm. female, December 30 (see Table 1). μ = 5,060 calories.

TEMPERATURE CHARACTERISTICS

It was surprising to find that an exceptionally rectilinear relation exists between the logarithm of the potentials and the reciprocal of the absolute temperature, as required by the Arrhenius equation:

$$ln \frac{R_1}{R_2} = \frac{\mu}{2} \frac{1}{T_1} - \frac{1}{T_2} .$$

It was assumed that each potential represents the *rate* of reaction of a process generating the electromotive force. Francis (1933) has demonstrated a continuous current of $20\text{–}30 \times 10^{-6}$ amperes maintained for many hours by frog skin. Of the total of thirteen satisfactory graphs, five yielded a μ-value in the vicinity of 5,000 calories; six yielded a μ of about 10,000 calories (see Figs. 2 and 3). One skin gave a μ of 5,000 above 15°C. and 10,000 below this critical temperature. The μ-value of one skin was much higher than all the others, i.e., 17,250 calories. Motokawa (1938*b*) reported a μ-value of 15,900 for the action current in frog skin stimulated through the cutaneous nerves.

DISCUSSION

The low μ-value of 5,000 calories is not common for other physiological processes (cf. Barnes, 1937); and Crozier (personal communication) suggests that it may be the result of parallel processes, one of which has a high μ but contributes only slightly, while the other may be a simple physical matter, such as ionic mobility. These skins with the low μ-value approach the ideal case of a reversible galvanic cell, as postulated by Lesser (1907), but the potential follows the absolute temperature logarithmically. The low temperature characteristic lends support to the theory that the electromotive force is closely associated with ionic diffusion (cf. Amberson, 1936; Motokawa, 1938*a;* Dean, 1939).

The μ-value of 10,00 calories in certain skins clearly indicates a chemical process as the master-reaction in these preparations. The value is close to the mode of 11,000, which has been associated with hydrolysis. This value occurs in many respiratory phenomena and perhaps represents hydrolysis preparatory to oxidation. The value of 17,000 is close to the mode of 16,800, which is identified with oxidation. Indeed, Krogh's data on the oxygen consumption of the frog yield a μ-value of 17,000 calories (cf. Crozier, 1924). The dependence of the potential on metabolism is shown by the inhibiting action of the following depressants: cyanide, ether, and chloroform (Engelmann, 1872; Alcock, 1907; Lund, 1926); urethane (Boell and Taylor, 1933; Duce, 1936; Ponder and Macleod, 1937); sulphide, carbon monoxide, amyl and butyl alcohol (Francis, 1934); bromacetic acid (Huf, 1936); iodoacetate (Francis and Gatty, 1938); and heavy water (Barnes, 1936, 1939). It has been known for some time that oxygen deficiency lowers the skin potential (Engelmann, 1872; Lund, 1926; Williams and Sheard, 1932; Francis, 1934; Taylor, 1935–36). Also, the striking increase in potential produced by eosin (Barnes and Golubock, 1938) is usually accompanied by an increase in oxidation.

It is possible that the oxidation of large organic molecules releases bound ions, producing a diffusion potential associated with the μ-value of 5,000. If the supply of ions is low, the master-reaction will be the precursor chemical factors indicated by the μ-values of 10,000 and 16,000. The response of the skin potential to temperature may be modified by the presence of Ringer's solution on the outside of the skin (normally in contact with water). Several workers have localized the potential on the outside (Alcock, 1907; Duce, 1936). The linkage between metabolism and potential is probably very complex. Francis (1933) has calculated that the electrical-energy output is from 5 to 10 per cent of the energy released by oxidation (assuming glucose is burned). Dean (1939) has suggested that the negative charge may be the bicarbonate ion produced in respiration. Dean and Gatty (1937) believe that the diffusion potential is complicated by the mosaic nature of the skin, local-action currents, and ionic adsorption.

The specific temperature characteristic undoubtedly depends on the physiological state of the skin preparation but does not seem to be correlated with weight, sex, or dorsal or ventral surface of the frog (see Table 1).

SUMMARY

1. The effects of slow changes in temperature on the potential of isolated frog skin in Ringer's solution indicate temperature characteristics of 5,000, 10,000, and 17,000 calories.

2. It is suggested that the low μ-value of 5,000 is associated with ionic diffusion, and the higher values with metabolic processes supplying ions as precursors of the potential.

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THE ACTION OF CALCIUM ON MUSCLE PROTOPLASM[1]

(One plate)

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TO A cellular physiologist, impressed with the essential similarity of protoplasm in all types of living material, it is rather surprising that the students of muscular contraction have made so little attempt to explain the behavior of muscle fibers in terms of their protoplasm. The muscle physiologist, adept as he is at chemical analysis and at various intricate types of physical recording, has hardly ever considered the fact that the protoplasm of the muscle cell is not markedly different from the protoplasm of less differentiated cells whose physical properties are easier to study. At the present time, although we know that various organic compounds react during or after muscular contraction, no one has been able to correlate any single chemical reaction with the visible fact of muscle-shortening. It is true that there has been some recourse to molecular theories, and at various times Astbury has urged that the contraction of muscle is due to a superfolding of myosin molecules (see, for example, Astbury, 1938).

However, on the whole, it seems clear that the direct attack on the problem of what makes a muscle shorten has not yielded any immediate hope of a satisfactory solution. It is the purpose of this paper to interpret the peculiar power of muscle protoplasm to shorten in terms of our information concerning other types of protoplasm.

According to views previously expressed (for summary see Heilbrunn, 1937, chaps. ix and xxxvii), cells typically consist of a cortex which is a gel, and a more or less fluid interior. Among its other constituents, the cortex contains calcium in a bound form. On stimulation calcium is released from the cortex and enters the cell interior. There it produces gelation or clotting. This clotting reaction is essentially the same type of reaction as that which occurs when naked protoplasm is exposed by tearing or cutting a cell (surface precipitation reaction). (For further details of the theory and for references to experiments which support it see Heilbrunn, 1937.) If the theory is correct, it is possible to divide the effects of stimulation into two stages: the first involves the release of calcium from the cortex, and the second assumes that calcium produces a reaction in the protoplasm similar to the surface precipitation reaction.

In how far is it possible to apply this theory to the muscle cell? The experiments to be described show that the muscle protoplasm is extremely sensitive to calcium ion and that entrance of calcium into the cell causes pronounced shortening (compare also, Chambers and Hale, 1932). This constitutes evidence in support of the second part of the theory. In a later paper evidence will be presented to show that the irritability of the isolated muscle fiber is, to a marked extent, dependent on the presence of calcium in the medium surrounding it. This, together with the earlier work of Ashkenaz (1938*b*), which showed calcium release on ultra-violet stimulation of isolated muscle fibers, will offer support for the first part of the theory.

Single muscle fibers were dissected out from the adductor magnus muscle of the frog

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[1] This paper is the first of a series on isolated muscle fibers. The work is receiving the support of the Rockefeller Foundation.

(*Rana pipiens*). The dissections were made for me by Dr. Eleanor W. Ashkenaz, who in the past few years has developed unusual skill in this technique. During dissection the muscles were immersed in buffered Ringer's fluid (for formula see Ashkenaz, 1938*a*). In order to free the fibers, they must be cut. At each cut end a plug forms, sealing the end of the fiber. Such plug formation has been noted by pathologists (see, for example, Weihl, 1874 [Weihl's paper may be consulted for earlier references]; Thoma, 1906; compare also Speidel, 1938). It is my belief that the plug which appears at the cut surface of the muscle fiber is due to a surface precipitation reaction of the same sort as that which occurs generally when cells are torn or broken (see Heilbrunn, 1937, chap. ix). Certainly the plug formation seems to be favored by calcium ion, just as the surface precipitation reaction is. And yet, whereas the surface precipitation reaction is prevented by the presence of oxalate, in the case of the muscle fiber, large plugs are formed when oxalate is present. This is a point of some interest and will be referred to again later.

When an isolated muscle fiber is placed in an isosmotic solution of calcium chloride (0.09 M), a sudden change occurs. The fiber shortens rapidly until in a minute or two it is only a fraction of its original length. Under the microscope the fiber, as it shortens, presents an unusual picture. Near each plugged end the cross-striations seem to flow into the plug, there to disappear. As this occurs, the plug lengthens, its substance increasing at the expense of the muscle protoplasm. The advancing plug is preceded by a region in which an increase in diameter of the fiber has occurred. There is, accordingly, from each end of the fiber a wave of thickening, followed by a wave of plug formation. The waves from opposite ends meet, and before long the whole fiber is transformed into a mass of brownish-black plug material thicker than the original fiber and wrinkled in contour. As the muscle protoplasm becomes bit by bit converted into plugs, it loses all irritability and dies. The whole process requires about 2 or 3 minutes for completion. Plate I is a series of photographs of an isolated fiber in an isosmotic calcium chloride solution. The individual photographs were taken at 10-second intervals. In order to show the entire length of the fiber, a low magnification had to be employed. As a result, the photographs show little detail. However, they clearly illustrate the shortening of the fiber and its transformation into plug material.

It is a simple matter to show that the progressive shortening of the muscle substance and its transformation into plug material is due to the entrance of calcium into the plugged cut ends of the fiber. If an isolated fiber is suspended over two small glass rods, then it is possible to apply a calcium chloride solution either to the cut ends or to the uninjured surface along the length of the fiber. If the ends are exposed, shortening and transformation of protoplasm into plug material goes on at about the same rate as it does when the entire fiber is immersed. On the other hand, if the calcium solution is applied along the sides of the fiber, with the ends in Ringer's solution, no rapid shortening occurs. Or rather, no shortening occurs unless the fiber has been injured in the course of preparation. If points of injury exist, these behave just as do the ends, and pluglike material grows out from each injured spot.

These observations indicate clearly that, whenever muscle protoplasm is exposed to calcium chloride, there is a rapid and extensive shortening along the length of the muscle fiber. The progressive course of this shortening was determined by a series of measurements made on individual fibers immersed in unbuffered 0.09 molar calcium chloride solution (pH approximately 6.0). At half-minute intervals, the outlines of the fibers were sketched with the aid of a projection apparatus. This made it a simple matter to measure the lengths of the fibers at each successive interval. In all, fifty series of measurements were made; but in order to conserve space, only half of these measurements are recorded in Table 1. The figures in the table were obtained by measuring outline drawings in millimeters. Inasmuch as the magnification produced by the apparatus was 40×, in order to obtain the true length in millimeters it is necessary to divide the figures given in the table by 40. In three of the twenty-five recorded experiments the shortening process was much longer than in more typical experiments. In these three cases the records for successive lengths of the fiber after 5 minutes' exposure are omitted from the table, and only the final length is recorded. For the twenty-five fibers whose measurements are given in the table, the percentage of final length in terms of the original length is 28 per cent; for the entire series of fifty fibers, the percentage was 27 per cent.

TABLE 1

EFFECT OF ISOSMOTIC $CaCl_2$ ON ISOLATED MUSCLE FIBERS

No.	ORIGINAL LENGTH IN RINGER'S SOLUTION (MM.)	LENGTHS AFTER IMMERSION IN $CaCl_2$ FOR—										FINAL LENGTH	TIME FOR COMPLETE SHORTENING		$\frac{\text{FINAL LENGTH}}{\text{ORIGINAL LENGTH}} \times 100$
		Minutes													
		1/2	1	1½	2	2½	3	3½	4	4½	5		Min.	Sec.	
1...	132	112	102	94	....	72	48	....	....	....	....	48	2	40	36
3...	152	103	72	59	35	....	....	....	....	....	....	35	1	40	23
5...	142	95	72	53	46	....	....	....	....	....	....	46	1	50	32
7...	147	119	105	82	70	60	44	....	....	....	....	44	2	45	30
9...	175	115	95	73	64	70	48	....	....	....	....	48	2	50	27
11...	100	....	86	72	66	44	....	....	....	....	....	44	2	44	44
13...	250	....	255	....	220	....	158	75	....	....	....	75	3	40	30
15...	188	148	124	105	88	79	69	65	40	....	....	40	4	....	21
17...	174	112	77	55	37	....	....	....	....	....	....	37	1	35	21
19...	166	102	81	52	35	....	....	....	....	....	....	35	1	55	21
21...	146	98	89	62	37	....	....	....	....	....	....	37	1	58	25
23...	171	152	125	112	114	96	81	70	55	46	....	46	4	5	27
25 ..	171	121	97	83	63	48	....	....	....	....	....	48	2	25	28
27 ..	152	116	87	63	30	....	....	....	....	....	....	30	2	5	20
29...	111	62	35	25	....	....	....	....	....	....	....	25	1	25	28
31...	140	....	67	47	28	....	....	....	....	....	....	28	1	40	20
33...	196	188	177	160	140	....	113	....	45	....	54	54	5	....	28
35...	262	....	212	192	180	161	157	142	138	128	120	56	10	30	21
37...	184	....	173	....	160	....	148	....	....	....	....	84	12	....	46
39...	170	....	83	63	52	50	38	....	....	....	....	38	3	....	22
41...	192	147	127	108	101	87	82	72	55	41	....	41	4	30	21
43...	143	104	77	....	60	57	46	....	....	....	....	46	3	....	32
45...	155	134	120	111	106	100	97	91	92	92	88	76	9	....	49
47...	180	132	....	75	82	71	60	46	....	....	....	46	4	....	26
49...	200	150	130	100	86	75	60	39	....	....	....	39	4	....	19

If isolated muscle fibers are immersed in hypotonic solutions of calcium chloride, they also shorten rapidly. Thus, in solutions of half the concentration of isosmotic solutions, or even in solutions of one-fourth the concentration of isosmotic solutions, shortening is very nearly as rapid as in the isosmotic solutions. Fibers were also placed in hypertonic solutions of calcium chloride. It was thought that such solutions would cause a more rapid and a more complete shortening than the isosmotic solutions. However, in strongly hypertonic solutions, although there was an immediate and rapid decrease in length, the shortening soon stopped, and there was little, if any, further effect. Table 2 shows the final shortened lengths of the fibers in terms of percentages of the original length for various strengths of calcium chloride solution. Each value represents an average of from five to ten determinations, except in the case of the isosmotic solution, in which all fifty determinations are averaged. With moderately hypertonic solutions (i.e., solutions of twice or three times the isosmotic strength) there is extensive shortening, but this becomes progressively less as the concentration of calcium chloride is increased. Sometimes, in strongly hypertonic calcium solutions, after a rapid initial decrease in length, the fiber may, after a minute or two, grow slightly longer. In Table 2 the shortest length of the fiber was taken in calculating the percentages.

TABLE 2

EFFECT OF VARIOUS CONCENTRATIONS OF $CaCl_2$ ON ISOLATED MUSCLE FIBERS

Concentration of $CaCl_2$	Number of Experiments	Average of $\frac{\text{Final Length}}{\text{Original Length}} \times 100$	Average of Time Required for Complete Shortening	
			Min.	Sec.
$\frac{1}{4}$×isosmotic	6	33	3	10
$\frac{1}{2}$×isosmotic	9	28	2	45
Isosmotic (0.09 M)	50	27	3	13
2×isosmotic	8	25	2	12
3×isosmotic	8	28	1	29
5×isosmotic	10	60	1	7
10×isosmotic	5	75	2	30

The fact that muscle fibers do not shorten as much in strong solutions of calcium as they do in weaker solutions is of considerable interest. It may be that osmotic factors are concerned—at least to some extent. However, there appears to be an interesting correlation with the fact that in the sea-urchin egg the surface precipitation reaction, although initiated by the calcium ion, is inhibited by strong concentrations of the same ion (Heilbrunn, 1930).

Strontium and barium chloride behave much like calcium chloride in their effect on isolated muscle fibers. In isosmotic solutions of strontium or barium chloride the fibers shrink in much the same way and in about the same time as they do in calcium chloride solutions. In isosmotic solutions of sodium or potassium chloride there is no shortening at all comparable with that produced by calcium chloride.

Magnesium chloride acts in a remarkable fashion. Some years ago, in attempting to explain the anesthetic behavior of the magnesium ion and its antagonism by calcium ion, I suggested that both ions acted in the same way, except for the fact that calcium was far more potent (Heilbrunn, 1934). It was argued that immersion of cells in solutions of magnesium salts would result in a replacement of the calcium ion in the cortex by the far less potent magnesium ion. That magnesium is far less potent than calcium in causing a surface precipitation reaction was demonstrated for sea-urchin egg protoplasm. The reaction was produced by calcium in great dilution. To give the same effect, a concentration of magnesium ion at least one hundred times greater was necessary. A similar relation exists in these muscle fibers. When fibers are immersed in magnesium chloride solutions of half the isosmotic concentration, instead of shortening rapidly, as they do in solutions of calcium chloride of similar strength, the fibers typically lengthen slightly. However, in solutions of five times the isosmotic concentration there is an extremely rapid shortening. These preliminary experiments with magnesium solutions will probably serve as the basis for further work in this laboratory.

One strange phenomenon remains to be discussed. That is the action of oxalate ion. It has been noted that, whereas calcium causes a rapid shortening of the muscle, sodium and potassium have no such effect. And yet, when fibers are immersed in solutions of sodium or potassium oxalate, they show a progressive shortening much like that observable in solutions of calcium chloride. It is true that the decrease in length is much slower. Thus, in an isosmotic solution of sodium oxalate it may require 15–25 minutes for a fiber to attain maximum shortening. Aside from the slower speed the oxalate effect resembles rather closely the calcium effect. In oxalate solutions the fiber shortens from the ends, the plugs progressively lengthening until finally all the muscle protoplasm has disappeared into them. Apparently oxalate produces and encourages plug formation much as calcium ion does, and this constitutes a strong contrast from the relation of oxalate to the surface precipitation reaction in typical cells. In all other cells which have been studied, the surface precipitation reaction is prevented by solutions of oxalate salts. Here, then, is a contradiction, for in the frog muscle cell, oxalate seems to act like calcium. Apparently it is a widespread contradiction. For in many types of living systems, in spite of the fact that oxalate removes calcium from solution, it may act like excess calcium in stimulating to activity. If we could explain this peculiar stimulating power of oxalate ion, such an explanation would remove one of the major difficulties in the way of rational explanation of a number of vital activities. For often enough, under slightly varying conditions, oxalate may act now as a stimulant and again as a preventer of stimulation.

I believe that a simple explanation of the calcium-like effect of oxalate ion may be possible. When a cell, such as a sea-urchin egg, is broken into an oxalate solution, the protoplasm immediately mixes with the oxalate solution, and no surface precipitation reaction occurs. But when a muscle cell is torn or cut, probably because of the presence of fibrils, the protoplasm does not exude, and no mixture of protoplasm and oxalate is possible. If now we assume that magnesium ion diffuses more rapidly through the muscle cell than does calcium—and this is not an unreasonable assumption—then in the interior of the muscle cell, at some slight distance from the torn end, the protoplasm would be increasingly rich in calcium as compared to magnesium. In other words, the calcium-magnesium ratio would increase. Such an increase in the calcium-magnesium ratio might well produce effects of the same sort as those produced by an increase in calcium concentration (compare Wilbur, 1939). The result would then be that the oxalate across a barrier could produce the same general type of effect as an increase in calcium ion concentration.

An attempt has been made to test this hypothesis by immersing muscle fibers in magnesium chloride solutions before subjecting them to the oxalate solutions. If the hypothesis is correct, previous exposure to magnesium should delay the action of oxalate.

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PLATE I

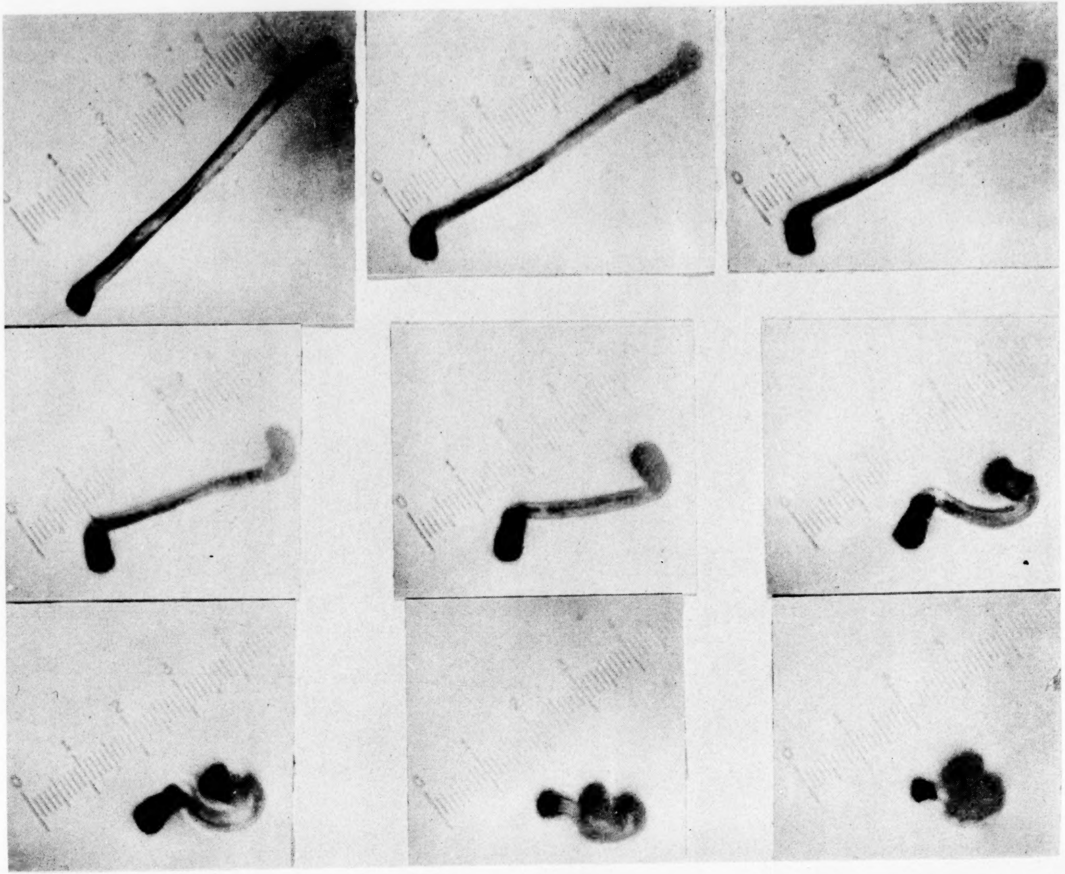

PLATE I

Shortening of an isolated muscle fiber in isosmotic (0.09 M) calcium chloride. The succession of stages is from left to right, as on a printed page. The first photograph was taken in Ringer's solution; the second, 10 seconds after the addition of calcium chloride. Subsequent pictures were taken at 10-second intervals.

The results obtained, although not striking, in general were in accord with the theoretical expectation, and it is hoped to publish them at some later date. However, it should be noted that the experiment is capable of other interpretations as well as that given by the proposed hypothesis.

SUMMARY

When isolated frog muscle fibers are immersed in calcium chloride solutions, they shorten rapidly and extensively. The calcium comes into contact with the muscle protoplasm by entering the cell through the cut ends of the fibers. Strontium and barium behave like calcium. Magnesium is effective only in relatively high concentration. The results are brought into relation to the colloid chemical theory of stimulation. An explanation is proposed for the strange fact that oxalate sometimes acts as though it were calcium.

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MITOTIC ELONGATION. II. OSMOTIC AND SALT EFFECTS

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WHEN a cell divides, it elongates. The nature of this elongation has never been completely analyzed, although recently Dan and his co-workers have developed a technique for investigating this process. By observing the behavior of kaolin particles adhering to the surface of cleaving sea-urchin eggs (species: *Mesipilia globulus*), they discovered that "the surface of the *cleaving* egg is the same as was covering the uncleaved egg, and that all increase in the measured distances up to this time is brought about by stretching" (1938, p. 528). What the physico-chemical factors may be that underlie this phenomenon of stretching remains unknown.

An earlier, and somewhat different, approach to this problem was made in 1936, when I determined the course of the elongation of the *Arbacia* egg at first cleavage. The data indicate that there are three distinct stages in the elongation process. The first is the "incipient period of elongation," which occurs *after* the diaster has formed (Fadda, 1924; Just, 1928) and is characterized by the progressive flattening of the surface of the cell in the equatorial region up to the time of the earliest appearance of the cleavage furrow. This phase is often loosely termed "karyokinetische Streckung." The second stage coincides with the rapid pulling-apart of the daughter cells during the indentation process. Actually, in the *Arbacia* egg, the blastomeres remain contiguous. The final stage is associated with the slow diminution of the extension resulting from the flattening against each other of the two blastomere surfaces that are in contact.

The present paper is an attempt to analyze from a colloid chemical viewpoint the first stage of the elongation process, described above as the "incipient period of elongation." Two types of experiments were carried out: first, the magnitude of this extension was determined under varying conditions of osmotic pressure; second, the qualitative effects of isotonic solutions of single cations were investigated.

METHODS

a) *Determination of the elongation.*—The measurements were made on *Arbacia* eggs divested of their fertilization membranes. Because of the short duration of the first stage of the elongation the method of measuring the extension was slightly different from that previously used. Instead of a filar micrometer, a camera lucida was used in conjunction with a special Zeiss objective (40×) of long working distance and a No. 4 Leitz ocular. With this setup rapid measurements could readily be carried out. Any single reading could be duplicated to within $\pm 0.5\ \mu$, so that a given measurement involved an error of about 1 per cent. During this brief period of measurement, which never exceeded 8 minutes, the temperature was constant to a fraction of a degree. Twenty measurements constituted one set, and for any given set the temperature varied less than 2° C. (24°–26° C.).

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[1] A thesis in zoölogy presented to the faculty of the graduate school of the University of Pennsylvania in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] I wish to thank Dr. L. V. Heilbrunn for his kind interest and aid during the course of this investigation.

b) *Hypotonic solutions.*—Eggs immersed shortly after fertilization in a solution whose osmotic pressure is less than 15.4 atm. (equivalent to a ratio of 70 parts of sea water to 30 parts of distilled water) do not elongate or divide. If, however, they are immersed just before the diaster stage, instead of at the beginning of the mitosis, then the physiological limit can be extended to a dilution of 60 parts of sea water to 40 parts of distilled water (osmotic pressure = 13.2 atm.). Osmotic equilibrium is reached rather rapidly in the spherical cell, as is shown by the fact that the measurements of the diameter attain a constant value soon after immersion.

c) *Hypertonic solutions.*—Hypertonic solutions of sea water were prepared by passing hot air through a flask containing sea water. The temperature did not exceed 85° C. By this method the bicarbonate-carbonate buffer system remains intact. The pH of these solutions, determined colorimetrically, showed no difference from that of sea water.

d) *Isotonic salt solutions.*—Isotonic solutions of the four common salts were used: 0.53 M NaCl, 0.53 M KCl, 0.29 M $CaCl_2$, and 0.29 M $MgCl_2$. Solutions of these strengths do not shrink or swell the eggs. (The pH of these solutions was usually between 6.0 and 7.0, i.e., much lower than that of sea water, pH = 8.2.) Six or seven washings, each with 200 cc. of the salt solution, were made at 5-minute intervals during the first cleavage.

RESULTS

a) *Osmotic effects.*—By varying the osmotic pressure of the external solution it was found possible to modify the degree to which the cell elongated before the furrow began to appear. The experimental data are presented in Table 1. In the second column are listed the experimental solutions which were used. These are expressed, in the case of hypotonic solutions, in terms of cubic centimeters of sea water plus cubic centimeters of distilled water. For hypertonic solutions figures are given in terms of 100 cc. of sea water less the volume removed by evaporation at high temperature. The osmotic pressures of these solutions are given in the third column. The pressures of the hypotonic solutions were calculated from the actual or interpolated values of Garrey's freezing-point data (Garrey, 1915). I am indebted to Jay Smith, of Johns Hopkins University, for the determination of the freezing-points of the hypertonic solutions.

The various values (in micra) for the extension of the cytosome before the appearance of indentation are given in the fourth column. The fifth column represents these extensions expressed as the percentage of the diameter of the egg in the experimental solution while still spherical.

The fertilized egg of *Arbacia punctulata*, with its fertilization membrane removed, elongates 3.3 μ (or 4.6 per cent of its diameter when measured along the long axis of the diaster) during the incipient period of first cleavage. This value has been given in italicized figures in the table. (Each value for the elongation represents the average of twenty experiments.)

The osmotic pressure of sea water is stated by Garrey to be 21.9 atm. Lowering the osmotic pressure by 2 atm. has little, if any, effect. The average of twenty measurements made on eggs in such a solution gives a total extension of 3.5 μ, which is only 0.2 μ

greater than the normal elongation. This increment lies within the limits of the experimental error. However, as the range of hypotonicity is extended, a very marked effect appears. Thus, for example, when the external osmotic pressure is reduced to 15.4 atm., the elongation has increased to 6.1 μ; i.e., the elongation increases with decrease in external osmotic pressure. This relationship is not quite linear but becomes transcendental in the limiting region.

An interesting observation is the fact that, in extremely hypotonic solutions (i.e., 1 part of sea water to 1 part of distilled water), the cells do not cleave but often remain elongated. At times the equatorial region of the cell bulges out. Of greater interest is the observation that there is a considerable accumulation of red-pigment granules traversing the interzonal body in the region where the furrow would have appeared ordinarily. In other words, a morphological demarcation has been partially effected in the absence of actual constriction. Whether this condition may be considered analogous to cell-plate formation in plant cells is questionable.

TABLE 1

THE EFFECT OF VARYING THE EXTERNAL OSMOTIC PRESSURE UPON THE VALUE OF THE INCIPIENT ELONGATION

	Composition of Experimental Solution in Cc. Sea Water ± Cc. Distilled Water	Osmotic Pressure of Experimental Solution (in Atm.)	Average Value of Incipient Elongation	
			Micra	Percentage
Hypertonic......	100−25	31.2	No cleavage	
	100−20	28.7	2.2±0.20*	3.3
	100−10	24.9	2.4±0.16	3.5
Sea water........	*100−0*	*21.9*	*3.3±0.16*	*4.6*
Hypotonic.......	90+10	19.7	3.5±0.11	4.7
	80+20	17.6	4.5±0.13	6.0
	70+30	15.4	6.1±0.18	7.5
	63+37	13.9	7.4±0.27	9.1
	60+40	13.2	8.7±0.28	10.4
	50+50	11.1	No cleavage	

* Probable error, $R = \pm 0.6745 \sqrt{\frac{\Sigma(v^2)}{n(n-1)}}$.

Hypertonic solutions, in contrast to hypotonic solutions, decrease the incipient elongation. A relatively small increase in the external osmotic pressure is sufficient to reduce the extension to almost the minimum. Thus, merely increasing the osmotic pressure by 3 atm. results in the cell elongating only 3.5 per cent over its original diameter, instead of the normal value of 4.6 per cent. An additional increase of 4 atm. (28.7 atm.) has hardly any further effect, the cell elongating 3.3 per cent. That the effects of hypertonic solutions are much more marked than those of hypotonic solutions is shown by the fact that in the latter the elongation can be increased from the normal increment of 4.6 per cent to as much as 10.4 per cent before the end-point is reached. This represents a physiological span of 8.7 atm. for hypotonic solutions, as against the span of 3 atm. for hypertonic solutions.

A comparison of the limits of osmotic pressure beyond which cleavage will not occur is interesting. Eggs do not divide in a solution whose osmotic pressure is less than 13.2 atm., which is 8.7 atm. less than that of sea water. In the other direction, eggs do not divide in a solution whose osmotic pressure is greater than 28.7 atm., an increase of 6.8 atm. The latter figure is considerably less than that reported necessary by Vlès and Dragoiu (1921*a*, 1921*b*) for the prevention of cleavage of the eggs of a European sea urchin. They state that the osmotic pressure can be raised by 11 atm. (calculated) before cleavage is impaired. However, considerable variation is indicated by the figures of Białaszewicz (1921). He gives the limits for the cleavage of *Echinus microtuberculatus* eggs as $\Delta = \pm 0^{\circ}.63$ and for *Strongylocentrotus lividus* as $\Delta = \pm 0^{\circ}.57$. These values may be compared with $\Delta = +0^{\circ}.73$ and $\Delta = -0^{\circ}.55$ reported here for the *Arbacia* egg.

b) *Salt effects.*—In the case of cation effects, careful distinction must be drawn between physiological and toxic actions. The latter, of course, have no meaning for this investigation. Thus, the sodium ion causes cytolysis, and its effect on the elongation cannot be studied.

Of the three remaining cations, potassium and magnesium permit incipient elongation; calcium prevents it. Whereas incipient elongation is favored by pure solutions of potassium and magnesium, nevertheless the furrowing process may be abnormal in these solutions. Ameboid motion, such as has been described by F. R. Lillie (1902) for the *Chaetopterus* egg, often sets in. Because the shape of the cell becomes ameboid, it is not possible to make quantitative measurements of the magnitude of the incipient elongation in these solutions. As the cell elongates, its shape becomes irregular, blebs form upon its surface, and indentations marking regions of furrow formation appear and disappear asymmetrically at various points on the cell surface. Internally, the ordered movements of the red-pigment granules toward the cell equator are disrupted, and clumping of these granules takes place in the interior of the cell as well as in the cortex. Many of these granules explode and release their pigment to the exterior. The behavior of the mitotic spindle in relation to the churning movements of the cytoplasm is especially interesting. It seems to bend and twist in every conceivable way. Its long axis may often be perpendicular to that of the cell, and it is not uncommon to find the mitotic figure restricted entirely to one of the two daughter blastomeres. The astral rays of the newly forming asters in the daughter cells are usually markedly bent. These observations fit in with the general conception that the spindle is a rigid body. Less frequently cases are met with in which the ameboid cells elongate to an abnormally great extent and then apparently "set" irreversibly. Nuclear divisions, as well as cytosomal cleavage, are entirely abolished. Such cells usually burst through their fertilization membrane.

Experiments with isosmotic KNO_3, KBr, and KI gave essentially the same picture, indicating that the effects are associated with the cation rather than with the anion. Similar results were obtained with isotonic KCl solutions made alkaline with KOH to pH 8.4 and made acid with HCl to pH 5.8. It is quite obvious that ions that favor cell extension may in themselves be detrimental to the cleavage process as a whole by effecting pathologically the mechanism of furrow formation.

Eggs immersed in an isotonic $CaCl_2$ solution remain quite spherical, showing no tendency to elongate. Also, there is no furrow formation; i.e., the entire cleavage cycle is reversibly inhibited. Nuclear division continues for some time.

DISCUSSION

There are many theories of the mechanics of cell division. For the older literature the critical review of Meek (1913) may be consulted. Teichmann (1903), in a widely quoted paper, postulated that cortical changes of a colloid-chemical nature were of prime importance. More recently Chambers (1938) and Marsland (1938, 1939) have especially emphasized the role of sol-gel transformations. As stated in the introduction, it is my purpose to analyze the situation from the same standpoint.

From this viewpoint a consideration of the results on cytosomal elongation obtained by immersing the eggs in isotonic salt solutions is of particular significance. This is because the effects of cations on the properties of colloids are very marked. As far as their action on protoplasmic colloids is concerned, salts have been found to act differently on different parts of the cell (Heilbrunn, 1937). Obviously, the reactions of the cell may be regarded as the physiological integration of various interrelated colloidal changes, and the contribution of any one factor to the end-result is extremely difficult to evaluate. Neglecting, for the moment, these general considerations, it is instructive to compare the effects of cations on the viscosity of the cell cortex with their effects on cell elongation. Data from both physiological and embryological sources are generally agreed that the calcium ion causes cortical gelation. Potassium, on the other hand, liquefies the cortex (Heilbrunn and Daugherty, 1932). Magnesium, although capable of forming nondialyzable proteinates (Duce, 1937*a*, 1937*b*), and so presumably likely to cause gel formation, has been found to liquefy the cell cortex (Heilbrunn and Daugherty, 1932). All that this may mean is that, compared to a calcium proteinate cortex, a magnesium proteinate cortex is relatively less viscous.

Evidently the colloid-chemical effects of these ions on the cell cortex exactly parallel their effects on the incipient elongation. I have shown that potassium and magnesium favor elongation, whereas calcium prevents it. From these results the tentative conclusion may be drawn that any agent that has a liquefying action on the cell cortex will permit elongation. Potassium and magnesium are such substances. Conversely, any agent that has a gelating effect on the cortex may be expected to prevent incipient elongation. Calcium falls in this category.

As a matter of fact, the parallelism between the known colloid-chemical effects of these ions on the cortex and their physiological actions can be shown even more strikingly. It has been found that, whereas pure solutions of potassium and magnesium favor incipient elongation, cleavage may be unsuccessful because the cells tend to undergo ameboid movements. This implies that, whereas agents that have a liquefying action on the cortex may favor incipient elongation, these same agents are not necessarily suitable for successful furrow formation. Evidently the furrowing process is not favored by cortical solation.

As a matter of fact, there is at present considerable evidence that the formation of the cleavage furrow is associated with a process of cortical gelation occurring in the equatorial region of the cell. Among such evidences, one may mention Schechtman's (1937) observations on vital staining during cleavage, Chambers' (1938) microdissection experiments, and Marsland's (1938, 1939) experiments on the effect of hydrostatic pressure upon furrow formation. Thus Marsland (1938, p. 63) writes that "the inhibiting effect of pressure upon the cell division is related to an interference with gelation processes which occur in the cortical protoplasm of the wall of the furrow rather than to other factors."

Experimentally, I find that the fewer the number of washings with isotonic solutions of potassium or magnesium the higher the percentage of successful cleavages. Evidently even traces of calcium in the sea water, carried over by incomplete washing, are sufficient to overcome the liquefying action of potassium and magnesium to the extent that furrow formation is normal. As a result, the number of cells exhibiting ameboid motions is considerably reduced. That small traces of calcium can be effective in counteracting the liquefying effects of potassium and magnesium is shown by the work of Heilbrunn and Daugherty (1932, pp. 265–66), where it is pointed out for the plasmagel of Ameba that "even 1 part of the calcium chloride solution to a 1,000 parts of the potassium chloride solution is enough to raise the centrifuge value from 20 to 35."

Clearly, two more or less interrelated processes occur in the cytosome during cell division. One is the process of cell extension, which apparently involves a liquefaction of the cell cortex in all regions of the egg other than the equatorial region. The other is furrow formation, which involves a gelation of the cortical protoplasm passing through the spindle axis. Bearing this general picture in mind, it becomes possible to analyze to some extent the experiments on varying the external osmotic pressure.

It has been shown that the action of hypotonic solutions is to increase the incipient elongation, and that of hypertonic solutions to decrease it. In regard to the action of hypotonic solutions, this statement is perhaps misleading, for these experiments, although intended to apply to only one process—incipient elongation—actually pertain to two processes: furrow formation and incipient elongation. It is evident, therefore, that the effect of hypotonic solutions in increasing the elongation may be due to one of two causes. In the first place, hypotonic solutions may *actively* cause the cell to increase in length above the normal value. On the other hand, the effect of decreasing the external osmotic pressure could very well be to cause a delay in the formation of the furrow. Obviously, anything that inhibits the development of the furrow without effecting the elongation mechanism would give higher values for the incipient elongation. The fact that the time required for the completion of the elongation process is greater in hypotonic solutions than in sea water indicates that the latter explanation is probably the correct one.

I conclude, therefore, that hypotonic solutions only indirectly increase the magnitude of the incipient elongation. The manner in which this takes place appears to be by an inhibitive action upon the onset of the constriction process. The fact mentioned previously, that in extremely hypotonic solutions the cell bulges out instead of constricting at the equator, is in line with the foregoing reasoning. Hypertonic solutions apparently act directly to decrease the extension. It does not seem possible, with our present knowledge, to offer an explanation of the quantitative aspects of these experiments.

SUMMARY

1. The value of the incipient elongation (so-called "karyokinetische Streckung") of the *Arbacia* egg at first cleavage has been determined under normal and experimental—variable osmotic and ionic—conditions. In sea water (osmotic pressure = 21.9 atm.) the cell elongates 3.3 μ (an increase of 4.6 per cent over its original diameter) before the cleavage furrow appears.

2. When the external osmotic pressure is decreased, the value of the incipient elongation is increased. This relationship is not quite linear. Eggs do not divide in a solution whose osmotic pressure is 11.1 atm.

3. Hypertonic solutions reduce the value of the incipient elongation, and eggs do not divide in a solution whose osmotic pressure is 31.2 atm. This indicates that cell extension is as important a factor in cleavage as furrow formation, since a furrow cannot form unless the cell first elongates.

4. Isotonic potassium and magnesium solutions favor incipient elongation; calcium inhibits it. Sodium is so toxic that its effect on the elongation could not be determined. The presence of calcium appears to be necessary for successful furrow formation, for eggs immersed in pure potassium and magnesium solutions exhibit ameboid motions and cleave imperfectly.

5. The data cited have been used as a basis for the interpretation of the physiology of cell division along colloid-chemical lines, as originally suggested by Teichmann many years ago. There appear to be at least two important factors in the division mechanism: elongation and constriction. Incipient elongation seems to be associated with the liquefaction of the cell cortex; constriction, with a gelation of the cortical protoplasm in the equatorial region. Hypertonic solutions act by arresting the elongation process. Hypotonic solutions probably inhibit the constriction mechanism. To a certain extent, elongation is independent of constriction; but the latter process depends upon, and becomes concomitant with, the former at the close of the incipient period of elongation.

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ENVIRONMENTAL FACTORS AFFECTING CYSTMENT IN WOODRUFFIA METABOLICA

(Five figures)

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CERTAIN aspects of cystment in the life-history of *Woodruffia metabolica* have already been described (Johnson and Evans, 1939). In that study it was pointed out that this ciliate regularly forms thin-walled cysts for division, that it forms thin-walled cysts in which no division occurs and in which the organism apparently digests its food, and that it regularly forms thick-walled resting or protective cysts in the absence of food. The purpose of the present investigation was to determine, at least in part, the causes of excystment from the resting state and the effects of various environmental factors on such excystment.

In the beginning of this study and throughout a large part of it, we assumed that all resting or protective cysts were alike to the extent that they required the presence of some organic substance in the excysting medium for excystment to occur. In the study referred to above, it was reported that a solution made by boiling dried *Elodea* leaves in balanced salt solution would consistently produce in the neighborhood of 100 per cent excystment. This is in agreement with the results of Taylor and his students in their work on *Colpoda*, where they have found that some organic substance, such as hay infusion or yeast extract, is necessary for excystment. Weyer (1930) reported that an organic substrate was necessary for excystment in *Gastrostyla*. Recently Haagen-Smit and Thimann (1938), in studies made on *Colpoda cucullus*, have found that the excysting activity of hay infusion is due to the salts of the simple organic acids present.

However, as a result of variations in the methods of preparing cysts, another type of resting or protective cyst was produced. This type of cyst can be activated without the addition of any organic substance. Other workers have reported excystment without the addition of any organic substance. Moore (1924) reported spontaneous excystment in *Spathidium*. Darby (1929) found that he could induce *Stylonychia* to excyst simply by adding fresh water to the cysts. Garnjobst (1937) obtained excystment in *Euplotes* upon the addition of tap or distilled water. Quite recently Taylor and Strickland (1938) have reported that cysts of *Colpoda duodenaria* may excyst spontaneously under certain conditions. The appearance of this second type of cyst in our work has necessitated an extension of our studies in order that a comparison of the two types of cysts might be made. The cysts which require the addition of some organic substance for excystment will hereafter be referred to as "stable" cysts. Those cysts which can be activated without the addition of some organic excysting substance will be called "unstable" cysts.

The two types of cysts differ characteristically in appearance, and it is possible to identify each type without question by low-power microscopic examination. Both types possess the same membranes—an innermost endocyst,[1] which is very thin; a mesocyst, which is quite rigid; and an outer, glutinous ectocyst. These membranes are shown in Figures 1 and 2. The endocyst is visible only after emergence from the other two membranes. It swells rapidly, and the organism escapes from it in a few minutes. At least one liquid vacuole is present in unstable cysts; such vacuoles are never found in stable cysts. The diameter of the protoplast of unstable cysts is always greater than the diameter of the protoplast of sister-cysts in the stable state. Such a comparison can easily be made in cultures started with a single *Woodruffia* in which both types of cysts develop. Owing to the volume difference, the granules in the cytoplasm of the unstable cysts are less closely packed than are those in the stable cysts. This causes a definite color difference in the two types when viewed under the microscope. These references to strictly morphological differences will serve as an introduction to the two types of cysts.

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[1] The use of "endocyst" instead of "intimocyst" for the innermost membrane, and "mesocyst" for the middle membrane instead of "endocyst," seems logical, although the other usage is common in the literature.

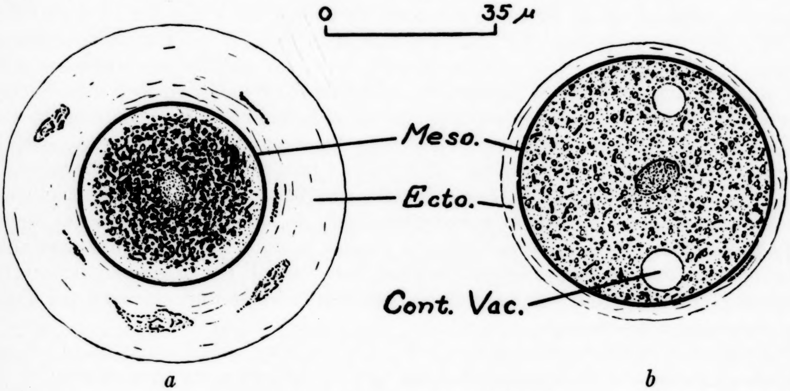

FIG. 1.—(*a*) Stable and (*b*) unstable cysts

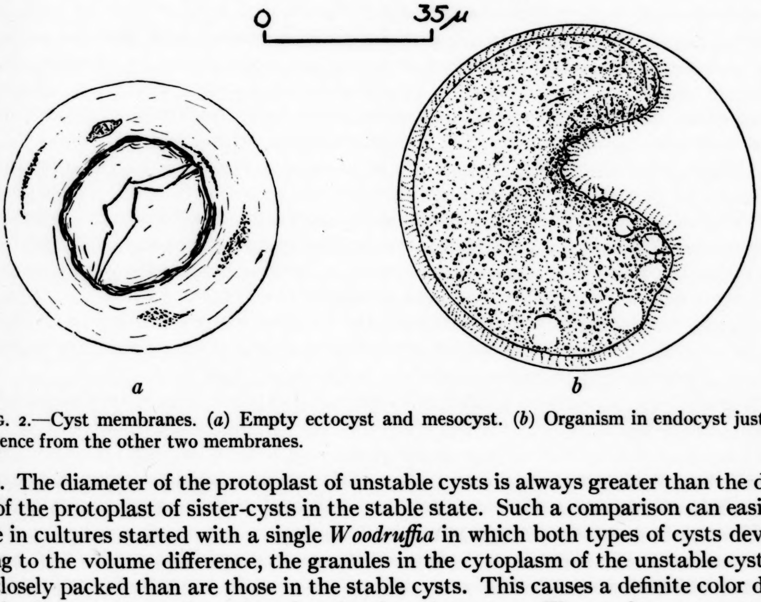

FIG. 2.—Cyst membranes. (*a*) Empty ectocyst and mesocyst. (*b*) Organism in endocyst just after emergence from the other two membranes.

MATERIALS AND METHODS

The experimental animals used in this investigation were obtained from a clone derived from the original sample collected on the Stanford campus (cf. Johnson and Larson, 1938). *Paramecium multimicronucleata* was used as a source of food. Some of the paramecia were grown in infusions prepared by boiling for 5 minutes $2\frac{1}{2}$ grams of dry timothy hay and 30 grains of wheat in 1 liter of tap water. Such infusion cultures of paramecia were kept in small wide-mouthed bottles. In addition to this source of food organisms, many cultures of paramecia, maintained in balanced salt solution (after Osterhout) with *Pseudomonas fluorescens* as the only source of food, were used. Thousands of paramecia were prepared for use daily by washing at least twice in fresh balanced salt solution by centrifuging. The washed paramecia were then transferred to Columbia watch glasses, and to these a few *Woodruffia* were added. In this way numerous stock cultures of *Woodruffia* were maintained. So long as food is present, division cysts and temporary or digestion cysts are greatly in the majority over free-swimming *Woodruffia*. Upon the depletion of the food, however, all the organisms become free-swimming for a short time. At this cultural phase *Woodruffia* were removed and washed, preparatory to encystment.

Balanced salt solution was prepared with triple-glass-distilled water and C. P. salts (Merck). Johnson (1933) has described the method for preparing the salt solution. Before use, this solution was buffered at a pH of 6.9–7.0. Experimental animals were always washed at least twice in the balanced salt solution.

Columbia watch glasses were used as experimental dishes. Following each experiment the dishes were thoroughly cleaned by scouring, rinsed with distilled water, and dried with a clean towel. Petri dishes were used as moist chambers. For experiments conducted at 15° and 20° C., constant-temperature rooms were used. For experiments conducted at all higher temperatures, incubators were used. Constant-temperature rooms were maintained at ±0.1° C., and incubators at ±0.5° C.

The method for inducing encystment in *W. metabolica* has been described by Johnson and Evans (1939). However, at that time only the so-called "stable" cyst was recognized, and only after subjecting the encysting organisms to a variety of experimental conditions was the unstable type encountered. The importance of standardization in the preparation of cysts is obvious. A discussion of factors involved in the production of unstable cysts will be given in a subsequent section of this report.

To insure the formation of stable cysts, the cultural history leading to encystment must be considered, though the factors existent at the time of encystment are of primary concern. The following procedure was followed in most cases.

Woodruffia, which had been feeding on large, well-fed paramecia, were washed twice in balanced salt solution by transferring with pipettes. Groups of approximately 30 *Woodruffia* were then removed to culture dishes containing 1 cc. of balanced medium. Unless otherwise stated, the total salt concentration of the balanced medium was 0.012 per cent, which will be designated as "X concentration." These *Woodruffia* were allowed to encyst at 25° C. to form standard cyst preparations.

It is necessary, for experimental purposes, to use cysts which are definitely mature, i.e., cysts which have completed the encystment process. In order to determine at what age cysts are mature, a series of dishes containing free-swimming organisms were prepared for encystment. After the organisms had rounded up and were in the first stage of encystment, they were placed in an incubator at 30° C. This was considered as zero time; and at regular intervals—namely, 24, 36, 48, 60, 72, and 84 hours—the salt solution was drawn from some of the dishes and the excystment medium added. The average time of excystment in the different dishes was then determined. These results were obtained:

Hours after start of encystment.....	24	36	48	60	72	84
Hours required for excystment......	27	10	8	7–7½	7–7½	7–7½

Under these conditions the cysts were mature after 60 hours. Similar tests were made with 25° cysts. In these the maturation time was somewhat longer, but in all instances the cysts were mature before the end of the fourth day. In all the excystment experiments, unless otherwise stated, no cysts were used which were less than 4 days old. The excystment time remained the same over a long period of time. The time required for 50 per cent of the organisms to excyst in any given experiment was considered as the excystment time.

In the beginning of these investigations a variety of solutions were tried as to their ability to induce excystment of stable cysts. Plant extracts, such as lettuce, timothy hay, lawn grass, and *Elodea*, would activate the cysts. Quite favorable results were obtained by the use of *Elodea* solutions. During the course of the work solutions of dehydrated alfalfa leaves were tried with excellent results. Solutions of yeast extract, which Taylor and Strickland (1935) reported as giving excellent results with *Colpoda* cysts, were not so satisfactory in this work. The yeast-extract solutions induced excystment, but the growth of bacteria was so much greater than in the other solutions tested that its use was discontinued. Solutions of various vitamins were tried without success. Bacterized gelatin, described by Weyer (1930) as being active on cysts of *Gastrostyla*, was also tried, without results, on cysts of *Woodruffia*.

Both *Elodea* and alfalfa extracts were used as excystment media in the experiments described below. The solutions were prepared by boiling for 3 minutes 3 grams of the dried plant in 500 cc. of buffered balanced salt solution. After filtering, the solutions were autoclaved in test tubes; and, when required, samples were removed with sterile pipettes. In this way the sterility of the solutions could be maintained indefinitely. Any variation in this procedure will be described in respective sections of the paper.

STABLE CYSTS

a) Concentration of the excystment medium.—As mentioned above, solutions of dried *Elodea* and of dehydrated alfalfa leaf meal were the best excystment media tried. Table 1 shows the average excystment results obtained in five trials using different concentrations of the two substances on standard cyst preparations.

These solutions were prepared by first making a 0.6 per cent infusion of each substance as described above. Parts of each stock infusion were diluted with balanced salt solution to the concentrations to be used. The test solutions were then autoclaved.

The results given in Table 1 show that the excysting substance must be present in certain concentration to produce maximal excystment and that below a certain concentration, in each case, no excystment occurs. In tests made earlier, solutions of dried *Elodea* in concentrations up to 1.0 per cent had been tried with approximately 100 per cent excystment in concentrations of 0.6–1.0 per cent. As a result *Elodea* solutions of 0.6 per cent were used in the earlier experiments. The results given here show that the excystment time is a little shorter in the alfalfa solutions and that below 0.6 per cent concentration the alfalfa solutions were more potent than similar concentrations of the *Elodea*. In some of the experiments reported below, a 0.6 per cent alfalfa solution was used; in others, a 0.6 per cent solution of *Elodea*. The kind of excystment medium used in each experiment will be stated. After the alfalfa was first tried, it was used in all later experiments. It is possible to obtain[2] large amounts of this dried leaf meal, thoroughly mixed and uniform; and in our work over the past six months no change has been detected in its potency.

Thimann and Barker (1934) state that the percentage of excystment in *Colpoda* is dependent upon the concentration of the hay extract used and is independent of the total amount present. Our results here confirm the first part of their statement concerning the concentration of the excystment substance. In our routine experiments we have varied the amount of the excystment medium from $\frac{1}{4}$ cc. to 1 cc. for a standard preparation, with no difference in results. However, to be able to say here that excystment is independent of the total amount of medium present, we would need to make further tests. Brown and Taylor (1938) found that yeast extract of a certain concentration was necessary to produce 100 per cent excystment of *Colpoda*.

TABLE 1

EFFECTS OF DIFFERENT CONCENTRATIONS OF *Elodea* AND ALFALFA AT 30° C.

ELODEA			ALFALFA		
Concentration (Per Cent)	Percentage of Excystment	Excystment Time (Hours)	Concentration (Per Cent)	Percentage of Excystment	Excystment Time (Hours)
0.6	98	7.25	0.6	100	6.5
0.4	80	7.5	0.4	98	6.5
0.2	74	7.5	0.2	95	6.5
0.1	55	8	0.1	84	6.5
0.06	21	8.5*	0.06	53	7
0.04	20	8.5*	0.04	34	7.25*
0.02	0	....	0.02	6	8.25*
0.01	0	....	0.01	0	....

* Where less than 50 per cent of the cysts were activated, the time given is for the total percentage of excystment.

b) *Effects of temperature on excystment.*—Variations in the temperature markedly affect the time of excystment in *Woodruffia*. The variation in the rate of excystment with temperature is shown in Figure 3.

The optimum temperature for excystment, as judged by excystment time, is 35° C. It was impossible to activate wet cysts at 15° C. Dry cysts, however, could occasionally be activated at this temperature, but the percentage of activation was low. Above 35° C. there is an increase in excystment time which is quite marked. As a temperature of 38° C.

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[2] The dehydrated alfalfa leaf meal was obtained from the Denver Alfalfa M. and P. Co., Lexington, Nebraska.

is lethal (cf. Johnson and Evans, 1939), it is quite probable that cysts activated near this upper temperature limit are injured and that such injury prolongs the excystment period.

Thimann and Barker (1934) found the optimum temperature for excystment of *C. cucullus* to be 24° C. The optimum for *W. metabolica*, as shown here, is considerably higher.

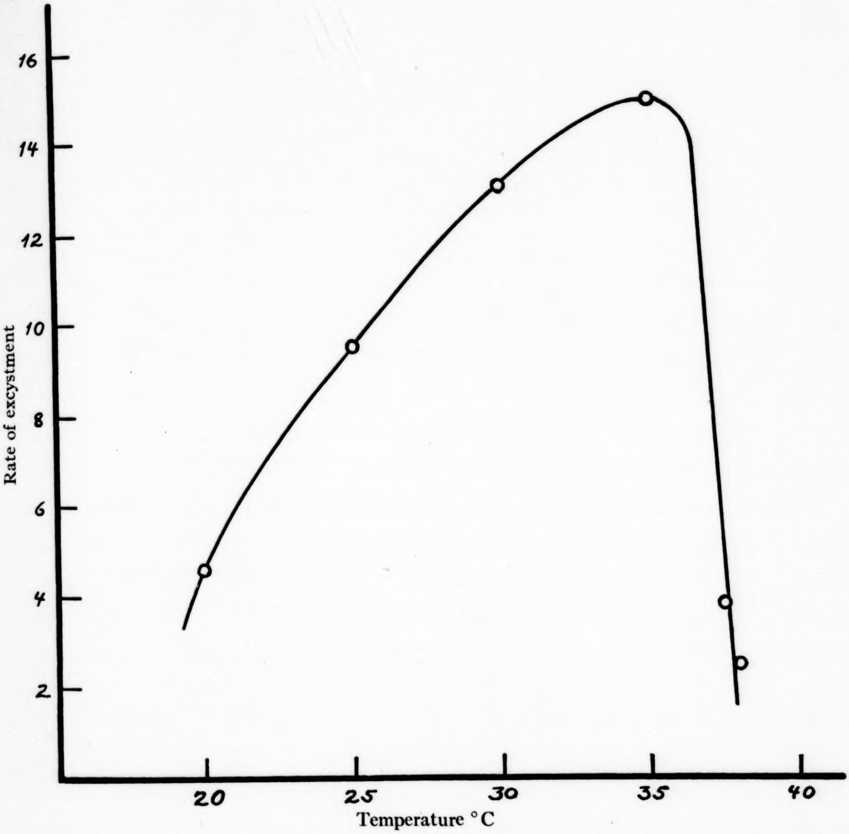

FIG. 3.—Effects of temperature on rate of excystment. The rate is expressed as the reciprocal of the time in hours times 100.

Various workers have found that resting cysts of a number of species of protozoans are quite resistant to extremes of temperature. Tests were made in these studies to determine the resistance of stable resting cysts of *Woodruffia* to extremes of temperature.

c) pH of the excystment medium.—Koffman (1924) and Darby (1929) have reported that changes in the H-ion concentration of the medium may induce both encystment and excystment in *Colpoda* and *Stylonychia*, respectively. Goodey (1913) and Bodine (1923) found that excystment of *Colpoda* occurred in an alkaline, neutral, or acid medium. Other workers, in recent investigations, have been unable to repeat the results of Koffman and of Darby, using the same and different organisms. To test the influence of H-ion concentration on the excystment of *Woodruffia*, three series of solutions (balanced medium, *Elodea* solution, and alfalfa solution), ranging in H-ion concentration from pH 5.0 through pH 9.0, were used on standard preparations of wet cysts at 25° and 30° C. The results are given in Table 2.

The results given here do not suggest that variations in the H-ion concentration of the medium, over a wide range, affect in any major way the excystment of *Woodruffia*. Excystment from the stable cyst does not occur at any H-ion concentration unless the excystment substance is present in sufficient concentration. The time required is slightly longer at pH 5.5. This concentration is near the acid death-point for free-swimming organisms, and Johnson and Evans (1939) found that cysts could not be formed in solutions more acid than pH 5.6. Readings taken at the end of these experiments showed that there had been some change in the pH of the alfalfa and *Elodea* media during the tests; e.g., media started at pH 5.0 gave readings of pH 5.5 at the end, and those started at pH 9.0 gave readings of pH 8.5, on the average. The time of excystment over the range studied is essentially the same. In an earlier paper it was shown that encystment occurs with the same facility over the same range of H-ion concentrations. The H-ion concentration of the medium becomes a limiting factor for both encystment and excystment of stable cysts of *Woodruffia* at pH 5.5–5.6. Dried cysts responded throughout the same range of H-ion concentration as that reported in Table 2 for wet cysts.

TABLE 2

HOURS FOR 50 PER CENT EXCYSTMENT AT DIFFERENT H-ION CONCENTRATIONS

TEMPERATURE OF EXCYSTMENT MEDIUM	pH OF THE MEDIUM								
	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
30° balanced salt*	....	....	....	....	....	....	....	....	....
30° *Elodea*	....	8.25	7.5	7.5	7.5	7.5	7.5	7.5	7.25
30° alfalfa	....	8	7	6.5	6.5	6.5	6.5	6.75	6.5
25° *Elodea*	....	11	10.5	10.5	10.5	9.5	9.5	9.5	9

* The preparations started in the balanced salt solutions showed no signs of activation after 48 hours. Addition of the buffered alfalfa solutions to these preparations caused activation and excystment in the normal time.

d) *Effects of salt concentration of the medium on excystment.*—Thimann and Barker (1934) state that they avoided the use of salt concentrations greater than 0.02 M in their studies on *Colpoda* because that organism is osmotically very sensitive. It has been shown that free-swimming *Woodruffia*, when placed in solutions of the Osterhout medium varying in total salt concentration from 0.012 per cent to 1.2 per cent, inclusive, will form viable resting cysts (cf., Johnson and Evans, 1939). Solutions with a total salt content of 1.44 per cent were lethal to most of the organisms. In these experiments differences in the viability of *Woodruffia* cysts, prepared in different salt concentrations and activated in different salt concentrations, were tested. The criterion used was the time required for 50 per cent excystment.

Two series of tests were made. In one, standard cyst preparations were made in salt solutions of each of the following concentrations: 0.012 , 0.024, 0.06, 0.12, 0.24, 0.6, and

1.2 per cent. When mature, these cysts were activated at 30° C. with the standard *Elodea* solution. In the other series standard cyst preparations were made in the usual way; and, when mature, these cysts were activated at 30 °C. with *Elodea* solutions made in the following salt concentrations: 0.012, 0.024, 0.06, 0.12, 0.24, 0.6, and 1.2 per cent. Figure 4 shows the results graphically.

Normal cyst preparations were activated in the same time, using excystment media varying in total salt content from 0.012 to 0.24 per cent. In fifty times the X concentration a somewhat longer time was required for excystment, and in one hundred times the X concentration excystment was considerably delayed. These results parallel fairly closely the results previously obtained on the effects of salt concentration on encystment.

Cysts which formed in the different salt concentrations and which were activated with the standard *Elodea* solution responded in the same time in the different preparations, with the exception of those formed in the 1.2 per cent salt concentration. These cysts were larger and had thinner cyst membranes than normal cysts, and excystment occurred in about 1 hour's time. This unusually rapid excystment was quite puzzling at the time, but it is now clear that they were the unstable type of cyst. A detailed discussion of unstable cysts formed in different salt solutions will be given in a later section.

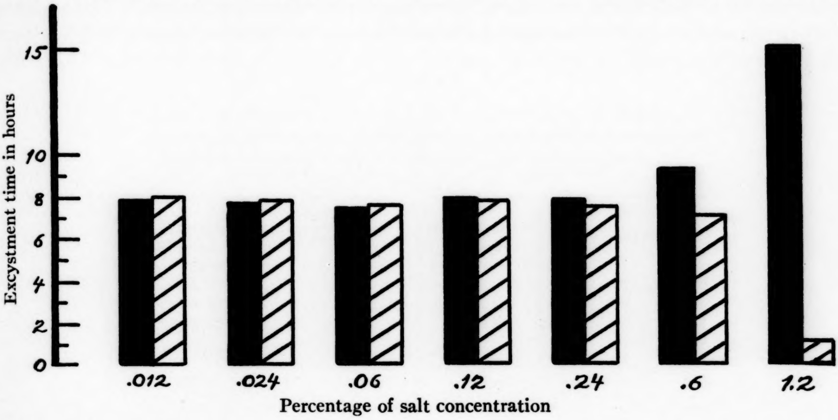

FIG. 4.—Effects of salt concentration on excystment. Solid bars, cysts formed in X salt concentration and activated at different concentrations. Cross-hatched, cysts formed in different salt concentrations and activated in standard *Elodea* solution.

These results show that normal resting cysts, as judged by the excystment time, may be formed and also may be activated, over a wide range of salt concentration of the medium. Weyer (1930) was able to obtain normal cysts of *Gastrostyla* over an even greater range of salt concentrations—from 0.006 to 2.0 per cent total salt concentration. In this study Weyer did not report the formation of cysts comparable to the unstable type reported here in the higher salt concentrations. In a number of different attempts it has been possible to induce the formation of normal *Woodruffia* cysts in the balanced salt medium with a total salt content as low as 0.003 per cent. Thus, the range in salt concentration of the medium in which this organism can form resting cysts is 0.003 per cent to 1.2 per cent, inclusive.

e) *Bacterized Elodea solution as an excystment medium.*—*Woodruffia* will form stable resting cysts in the *Elodea* solution and in other solutions which may be used as excystment media. The question arises: Do the bacteria which grow in such solutions in some way inactivate the substance or substances which cause excystment? To test this, some *Elodea* solution was bacterized with mixed bacteria from a hay infusion and then incubated at 25° C. for 3 weeks. At the end of this time the solution was filtered through a Chamberland filter to remove the bacteria. It was then used as excystment medium on standard cyst preparations. The excystment which resulted in five tests was normal both as to percentage of excystment and as to excystment time. In other words, excystment with this medium was quite similar to that obtained in control preparations in which sterile *Elodea* solution, prepared at the same time and incubated, was used.

If bacterial action does not alter the excysting potency of such solutions, why do cysts formed in such media remain in the encysted state over long periods? Thimann and Barker (1934) found that mixing encystment medium of *Colpoda* cultures with their excystment medium caused a reduced percentage of excystment. They say (p. 47): "Medium in which encystment has occurred in some way reduces the sensitivity of the organisms to the excystment stimulus. The action is upon the organisms themselves and not upon the excysting substance." The evidence presented here indicates that bacterial action does not inactivate the excystment substance. Further studies are needed to show just how the encystment medium changes the sensitivity of the organisms to the excystment stimulus.

TABLE 3

EFFECTS OF MOMENTARY EXPOSURE TO HIGH TEMPERATURES

DRY CYSTS		WET CYSTS	
Temperature (° C.)	Percentage of Excystment	Temperature (° C.)	Percentage of Excystment
100	100	45	87
110	97	48	85
120	100	51	86
125	92	54	88
130	0	57	0
135	0	60	0

f) *Resistance to extreme conditions.*—Bodine (1923) found that dried *Colpoda* cysts could withstand a temperature of 100° C. (higher temperatures were not tested) and that wet cysts, gradually heated, were viable after a momentary exposure at 55° C. Taylor and Strickland (1936) confirmed and extended this observation in their studies on *Colpoda*. They found that the upper critical temperature for wet cysts with momentary exposure was 51° C. and for dry cysts with momentary exposure was 121° C. Similar tests were made on wet and dry cysts of *Woodruffia*. The wet cysts were 4-day-old cysts; and the dried cysts, which had formed on Cellophane, were dried at room temperature for 2 weeks. Taylor and Strickland (1935) found that *Colpoda* cysts dried on Cellophane retained their viability longer than cysts dried on any other substrate. The results are given in Table 3.

Dishes of dried cysts were placed in an oven; and as soon as the temperature reached each interval to be tested, a dish was removed from the oven. From these results it appears that the upper lethal temperature for dried *Woodruffia* cysts is between 125° and 130° C. Dishes containing the wet cysts were placed in a water bath, and the liquid surrounding the cysts was constantly stirred with the bulb of a thermometer until the desired temperature was reached. The dishes were removed from the bath, and the cysts tested for excystment. The upper lethal temperature for wet *Woodruffia* cysts is between 54° and 57° C. These results are quite comparable to those obtained by other workers on the resistance of *Colpoda* cysts to high temperatures.

The question arises: When do such cysts begin to have this resistance to high temperatures? It has already been shown that 40° C. is lethal to the free-swimming organism. To test this matter, several standard cyst preparations were started at room temperature. As soon as the organisms had rounded up, one dish was placed in an incubator at 40° C. Following this, at intervals of 11, 19, 28, 35, 45, and 60 hours, other dishes were placed in the incubator at 40° C. In all the preparations, up to those transferred at the thirty-fifth hour, the organisms were still rotating quite rapidly within the cyst wall. After 4 days these cysts were removed and tested with the alfalfa solution for excystment. In the dishes transferred to 40° C. at the beginning and after 11, 19, and 28 hours, no excystment occurred. In all the other dishes excystment was over 90 per cent and in the normal time. These cysts, then, become resistant to a temperature which is lethal to the free-swimming organism after encysting at room temperature for about 35 hours.

TABLE 4

EFFECTS OF FREEZING ON STABLE CYSTS

Period of Freezing	Excystment Time	Percentage of Excystment
Control	7.5	98
24 hours	8.0	90
1 week	8.3	85
2 weeks	11.0	91
2 months	9.0*	20

* Excystment time refers to hours for 50 per cent excystment in each case except for those cysts frozen for 2 months. Here the excystment was only 20 per cent, and the time given is for that percentage.

Bodine (1923) found that cysts of *Colpoda* were viable after freezing. Taylor and Strickland (1936) obtained some excystment from preparations of *Colpoda* cysts which had been exposed to liquid-air temperature for 12½ days. The effects of freezing on *Woodruffia* cysts are given in Table 4. Dishes containing 4-day-old standard cyst preparations were placed in the ice chamber of an electric refrigerator. The medium surrounding the cysts was frozen in 2 hours. At the end of each time-interval to be tested, cyst preparations were removed to room temperature for 24 hours and then tested with *Elodea* solution for excystment.

The results given here are the averages of three preparations in each case except for those cysts frozen for 2 months. Freezing for 2 weeks has little effect on the viability of these cysts, but the excystment time was somewhat lengthened. After 2 months of freezing, however, in the one test made, the percentage of viable cysts was considerably reduced. Bodine (1923) referred to the fact that similar protozoan cysts regularly undergo long periods of freezing in certain regions. So it seems probable that further testing might reveal that these cysts could withstand even longer periods of freezing, and, perhaps, with even higher percentages of viability than was obtained in this one test.

In addition to being able to withstand extremes of temperature, some protozoan cysts will remain viable for extremely long periods of time in the dried condition. Dawson and Hewitt (1931) report that *Colpoda* cysts could be activated after being in the dried state for 5 years and 4 months. Taylor, in this laboratory, has kept dried *Colpoda* cysts for an even longer time without complete loss of viability. Beers (1937) reviewed the literature on the viability of dried cysts of different protozoa over long periods of time. In his studies he found that, although *Didinium* cysts were not viable after drying, some cysts were still alive after 10 years in hay infusion. The indications are that *Woodruffia* cysts are comparable to *Colpoda* cysts in their ability to withstand drying over long periods. The effects of aging on viability, in the tests made to date, are given in Table 5. The dried preparations used here had encysted on Cellophane.

TABLE 5

EFFECTS OF AGING ON VIABILITY OF CYSTS

DRY CYSTS		WET CYSTS	
Age in Days	Percentage of Excystment	Age in Days	Percentage of Excystment
151	90		
372	85	372	85
516	65		

Standard 4-day-old preparations give from 95 to 100 per cent excystment. With aging, there was some falling-off in percentage of excystment, but after more than a year, 85 per cent excystment was obtained in both wet and dry cysts. Taylor and Strickland (1935) found that there was a gradual decrease in the viability of *Colpoda* cysts during the first 6 months after formation to about 75 per cent, and that the viability remained at this level for three more months. They also found that the time required for excystment was increased with the aging of the cysts. That is also true for *Woodruffia* cysts. Dry cysts, over a year old, required approximately three times as long for excystment as standard control preparations; and wet cysts, of the same age, required twice as long for excystment as control preparations.

The problem of whether protective cysts in a thoroughly dried condition carry on any metabolic activities is an interesting one. Darby (1929, p. 29) says, in a discussion of his methods of determining the viability of cysts of *Stylonychia*, that "the third [method] was by observing their respiration in the dry state by using Parker's method of CO_2 determination"; but he gives no further details of this test. To our knowledge, no one has yet reported a really critical test on this matter.

UNSTABLE CYSTS

"Unstable" resting cysts have already been defined as cysts which can be activated without the addition of some organic excystment substance. Cysts which behave in this way were first encountered in our studies when *Woodruffia* were induced to encyst and excyst seven successive times without feeding. After the third excystment, such cysts could be activated by merely changing the salt solution at room temperature (cf. Johnson and Evans, 1939). This behavior, although not explained, was attributed to the small size and starved condition of such cysts. In a later study, the original purpose of which was to prepare cysts at 30° C. and determine whether there was any difference in the excystment time of such cysts and the standard preparations formed at 25° C., cysts were obtained which could be activated by changing the salt solution. Four dishes, each containing 30 *Woodruffia*, were placed in an incubator at 30° C. After 4 days two of these dishes were removed to test for excystment. Considerable evaporation had occurred in these preparations, probably because insufficient water had been added to the Petri dishes for the higher temperature. The alfalfa solution was added to one dish, and fresh balanced salt solution was added to the other as a control. In the earlier experiments on 25° cysts salt controls were run for numerous trials with no excystment. In both of these preparations 50 per cent excystment occurred in much less than the average time required at 30° C. The other two preparations were then tested in the same way. An examination of these cysts showed that from 75 to 80 per cent of the cysts in each dish were large and thin-walled. These thin-walled cysts excysted in approximately 75 minutes in each dish. The thick-walled cysts never excysted in the control dish, but in the dish to which alfalfa solution had been added they excysted in from 6½ to 7 hours.

Our efforts were then directed to finding the factors which cause the formation of such cysts.

a) *The formation of unstable cysts.*—In the first attempts to obtain such cysts again, poor results were obtained. Out of 56 trials at 30° C., in which the organisms were prepared in the same way in which all standard cyst preparations had been prepared, unstable cysts were obtained in only 16 of the trials, with an average formation for the 56 trials of 2 per cent. To prevent evaporation of the solutions containing the cysts in these tests, precautions were taken by placing the Petri dishes in large moist chambers. In this way a double insurance against evaporation of the culture medium was provided.

The microscopic appearance of the unstable cysts suggests that there are fewer granules in the protoplasm of such cysts than in stable cysts, or that the granules in the unstable cysts are more diffuse. It was thought that the use of organisms containing fewer granules might produce the unstable type of cyst in higher percentages. Such animals can be obtained by delaying encystment. Delay in encystment may be caused by agitating free-swimming organisms when they form clumps to start encystment. By carefully watching preparations containing organisms ready for encystment, sucking up into a pipette the clumps which formed, and then ejecting them back into the culture dish, encystment was delayed from 4 to 5 hours. Such preparations were then allowed to encyst at 30° C. Out of 28 trials, unstable cysts were formed in only 7 of them, and in these instances very few such cysts were formed. The average percentage for all of these trials was 0.7 per cent. The stable cysts formed appeared to have thicker cyst membranes than usual. So, instead of facilitating the formation of unstable cysts, this treatment hindered it.

One preparation of cysts formed at 30° C., in which the *Woodruffia* used had been feeding on paramecia in a semistarved condition (these paramecia had been washed and crowded in the balanced salt solution for a week without food), had 20 per cent of the cysts of the unstable type. Thirty-six similar tests were then made, and in 23 of them unstable cysts were formed. The percentages of unstable cysts formed in these tests ranged from zero to 62 per cent, with an average for all of the trials of 9.6 per cent.

In another series of tests 5X salt concentration was used for the encystment medium. Out of 20 trials, unstable cysts were formed in 17 of them, with an average percentage for all of the trials of 18.7 per cent. These two series of tests show that both the use of organisms which had fed on semistarved paramecia and the use of encystment medium with a higher salt concentration increased the formation of unstable cysts.

In all of the tests referred to above, the preparations were made by starting 30 *Woodruffia* in ½ cc. of encystment medium. Other tests were made at 30° C. in which 300–400 *Woodruffia* were allowed to encyst in 1/20 cc. of medium. The *Woodruffia* for these tests were concentrated by centrifuging. To prevent evaporation in these cultures, large cover glasses were sealed over the depressions of the Columbia culture dishes. In 11 such tests, in which the *Woodruffia* used had been feeding on well-fed paramecia, 14.9 per cent of the cysts formed were of the unstable type. In 7 other tests, using *Woodruffia* which had been feeding on semistarved paramecia, the average formation of unstable cysts was 40.9 per cent. These results indicate that crowding may be a factor in causing the formation of such cysts and that the effectiveness of crowding in this connection is increased when coupled with the use of organisms which have been existing on a qualitatively deficient diet.

In only a few of the tests described above did the percentage of unstable cysts formed equal that of the four preparations in which this type of cyst was first noticed. It seemed desirable to be able to induce the formation of such cysts more consistently and in higher percentages. The fact that the smallest percentages of unstable cysts obtained in these tests at 30° C. were obtained in the tests where encystment was delayed, together with the variations obtained in the other tests, suggested that a disturbance of the organism in some critical period just prior to encystment might affect the ensuing encystment process. It is altogether possible that the organisms isolated for the tests were not always in exactly the same state with reference to encystment. To obtain cyst preparations without any disturbance of the organisms just prior to encystment, a series of experiments were run which will be referred to as "completion cultures." In these experiments paramecia were washed twice by centrifuging, and ½ cc. suspensions of such paramecia (averaging 2,000–3,000 per cubic centimeter) were placed in culture dishes with one washed *Woodruffia*. Such cultures were then incubated at 25° and 30° C. for 9 and 7 days, respectively, and were then examined and tested for unstable cysts. The number of cysts formed in these preparations varied from 1,000 to 1,500. At 25° C. the *Woodruffia* required about 5 days to eat all of the paramecia and begin encystment; at 30° C. they depleted the food and started encystment in 3 days. The average results of these experiments are given in Table 6.

The differences between well-fed and semistarved food organisms have already been described. The results obtained here were consistent, and the percentages of unstable cysts much higher than those in the other tests. It is evident from these experiments that unstable cysts may form at 25° C. under certain conditions, although they are not formed in uncrowded preparations in the X salt concentration at this temperature. The effects of using *Woodruffia* which had fed on semistarved paramecia was quite marked in these experiments. Also, consistently higher percentages of unstable cysts were formed at 30° C. than at 25° C. This difference at the higher temperature may be due in part to some effect of the higher temperature on the food organisms over a period of days and also to some difference in evaporation of the culture medium at the higher temperature. That evaporation has an effect was again evidenced in these experiments in two dishes not placed in the second moist chamber. In these two preparations formed at 30° C., in which the food of the *Woodruffia* prior to encystment had been well-fed paramecia, 100 per cent formation of unstable cysts occurred. However, in these two cultures evaporation had taken place almost to the point of dryness. These two preparations were not included in the results given in Table 6.

One series of six completion cultures was run at 25° C. in which 5X salt solution was used as the encystment medium. In these tests the *Woodruffia* which had been feeding on well-fed paramecia formed 45 per cent of the total cysts of the unstable type, while the *Woodruffia* which had been feeding on semistarved paramecia formed 96 per cent unstable cysts. These percentages are higher than those obtained in similar cultures at 25° C. using the standard salt solution. Other tests, using still higher salt concentrations, indicated that the percentage of unstable cysts could be raised by increasing the salt concentration of the medium. It was previously shown that *Woodruffia* forms viable cysts in salt concentrations up to 1.2 per cent. Six different experiments were conducted at 25° C., in each of which 30 *Woodruffia* were allowed to encyst in 1 cc. of balanced salt solution of each of the following concentrations: 0.012, 0.12, 0.6, 0.9, and 1.2 per cent. The results obtained in these experiments were quite consistent, and the averages are given in graphic form in Figure 5.

TABLE 6

FORMATION OF UNSTABLE CYSTS IN COMPLETION CULTURES

	TEMPERATURE			
	30° C.		25° C.	
	Well-fed Food Organisms	Semistarved Food Organisms	Well-fed Food Organisms	Semistarved Food Organisms
Trials	15	10	16	7
Average percentage of formation	76.6	94.4	30.6	53.9

High percentages of unstable cysts were obtained in the experiments in which only one factor was varied—the salt concentration of the encystment medium. In the other tests evidence was obtained which indicates that crowding of the organisms, feeding the organisms on semistarved paramecia prior to encystment, and increasing the temperature may induce the formation of unstable cysts in varying percentages. In the completion cultures at 30° C. where all of these factors were involved, 94 per cent of the cysts formed were of the unstable type; yet in uncrowded preparations in 100× salt concentration at 25° C., 91 per cent of the cysts formed were of this type. Also, 100 per cent formation of the unstable cysts occurred in two preparations in which evaporation, almost to dryness, occurred. Further studies are being made to determine whether such cysts can be formed under other conditions. At present it appears that more than one factor, either independently or cumulatively, may operate in causing the formation of unstable cysts. It is conceivable that all of the factors dealt with here might be operative in nature.

b) *Methods of activating unstable cysts.*—Reference to the fact that these cysts may be induced to excyst merely by changing the salt solution on them has already been made. Triple glass-distilled water was just as effective. In all cases in which the encystment medium was replaced by fresh salt solution or distilled water, activation of the unstable cysts was approximately 100 per cent. In several of the preparations some of the activated organisms, although they were able to break out of the mesocyst, were trapped in the ectocyst. In such cases the mesocyst appeared folded against the ectocyst. After a time these organisms would re-encyst. Only rarely are organisms in stable cysts trapped in the ectocyst—after activation. In two cases organisms trapped in the ectocyst of stable cysts have been observed to divide and then to re-encyst.

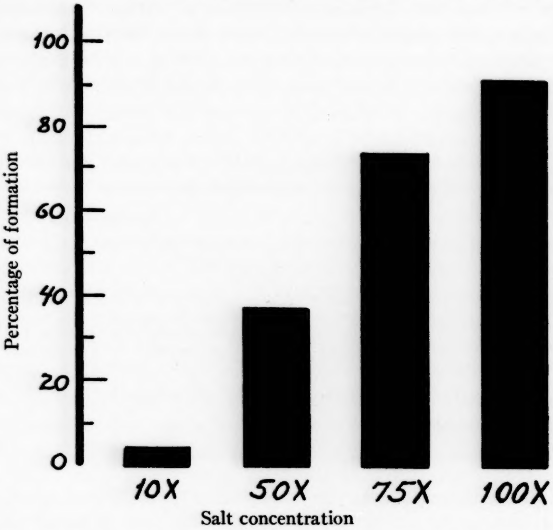

FIG. 5.—Formation of unstable cysts with increased salt concentration

By accident two preparations of unstable cysts were left on the laboratory table without changing the salt solution when they were removed from the 30° incubator for the excystment test. When they were examined 2 hours later, all the unstable cysts had excysted. Numerous other trials were made, removing preparations of the cysts from the 30° incubator to a temperature of 20° C. and also to room temperature (23°–24° C.) without changing the medium on the cysts. In all such tests the drop in temperature was just as effective in inducing excystment as changing the medium on the cysts. Here is an instance where a process of activation may be induced by a lowering of the temperature, which is unusual. Precisely what is involved here is not clear, and further studies are being made on the nature of this activation. That a very delicate balance exists between such cysts and their environment is obvious because, by either replacing the encystment medium with the inorganic salt solution or with distilled water or by simply lowering the temperature, they can be activated.

The excystment time of unstable cysts is quite short, compared to that of stable cysts. Unstable cysts formed at 30° C. and excysted at room temperature require about $1\frac{1}{4}$ hours for the process, while stable cysts under the same conditions require 12–15 hours. Many of the unstable cysts will excyst in 45 minutes. In every case where such cysts were examined under a compound microscope just after they had been changed to fresh salt solution, some cyclosis in the protoplasm was noticed, and in a short time the vacuole or vacuoles present began to function.

c) *Effects of aging and extreme conditions on unstable cysts.*—A series of completion cultures were started at the same time at 30° C. to use in weekly tests on the viability of unstable cysts. Several such tests have been made—the first, 2 weeks after the start of the cultures; and the others, weekly thereafter. Approximately 100 per cent excystment of the unstable cysts occurred in each test. This indicates that there is no change in the viability of these cysts for a period of more than 2 months. Additional tests on the effects of aging will be made.

Freezing for 24 hours did not affect the viability of unstable cysts. After thawing at room temperature all such cysts were activated by changing the salt solution on them. Luyet and Gehenio (1938) found that ice crystals did not form in the cells of moss leaves when their water content was 30 per cent or lower, and they imply that ice crystals will not form in cells with such a low water content. It seems probable that the unstable cysts of *Woodruffia* have a water content below the level where ice crystals will form, although they quite obviously have a higher water content than the stable *Woodruffia* cysts.

On the other hand, thorough drying is lethal to the unstable cysts. The encystment medium on several preparations was allowed to evaporate gradually over a period of 2 days in the 30° incubator. The first excystment test was made just as soon as all visible traces of moisture were gone. In this test 2 per cent of the cysts were activated. Ten other tests were made on successive days with no activation. Microscopic examination of these thoroughly dried cysts showed that the thin cyst walls were quite wrinkled and collapsed in appearance. Death, in these cysts, probably resulted from pressure on the protoplasts due to the distortion of the cyst walls during drying. The thick walls of stable cysts are not so affected by drying.

DISCUSSION

The results presented here on the effects of environmental factors on stable cysts of *W. metabolica* are similar in many respects to the results obtained in this laboratory by Taylor and his associates in carefully made experiments on cysts of *C. duodenaria*. Stable cysts of *Woodruffia* behave much like cysts of *Colpoda* in the following regards: (1) the presence in the medium of some organic substance above a certain concentration is necessary for excystment, (2) the time required for excystment is dependent on temperature and, within certain limits, the excystment time is shorter the higher the temperature, (3) over a wide range the hydrogen ion concentration of the medium does not affect excystment, (4) freezing for long periods does not lower greatly the percentage of viable cysts, and (5) such cysts can withstand thorough drying for long periods of time.

The discovery of a second type of protective cyst, the unstable cyst, in *Woodruffia*, which forms under certain conditions almost 100 per cent and which differs from the stable type both morphologically (in that the ectocyst is much thinner, the protoplast larger, the granules more diffuse, and a vacuole present) and physiologically (in that no organic substances are required for excystment and in other ways), raises a number of interesting questions.

As mentioned above, it is not clear at the present time whether several factors working independently operate to produce the unstable cysts or whether there is some underlying single factor which causes the formation of such cysts. The facts that crowding, increasing the temperature, and increasing the salt concentration of the medium tend to increase the percentage of such cysts and that a mere lowering of the temperature will activate them suggest that changes in oxygen tension may be involved both in the formation and in the activation of these unstable cysts. Tests are now being made to determine if such relationships exist.

It has been recognized (Taylor, 1935) that a more or less thorough dedifferentiation of the protoplasm occurs during encystment. Garnjobst (1937) has reviewed the literature on this subject, pointing out that differences in degree of dedifferentiation occur both within a single species and between species. While no studies have been made on the differences between the stable and unstable protective cysts of *Woodruffia* as regards dedifferentiation, certain things are suggested. The presence of a vacuole in the unstable cysts is indicative of less dedifferentiation than that in stable cysts. Cyclosis or motion in the protoplast of unstable cysts is noticeable when examined under a microscope immediately after their removal from a preparation. Definite rotation within the cyst wall occurs after several minutes, and the whole process of excystment is often completed in 45 minutes. In contrast with this, stable cysts require about 6 hours for excystment under the most favorable conditions. These differences also suggest that the unstable cysts are in a less dedifferentiated state than stable cysts. The factor or factors which induce the formation of unstable cysts seem to inhibit the secretory processes involved in the formation of the ectocyst, and it appears likely that the process of resorbtion and dedifferentiation is also inhibited, or at least in part. Garnjobst (1937, p. 369) found that, "of the two hypotrichs under consideration (*Stylonethes* and *Euplotes*), the cysts which withstand drying show a greater degree of dedifferentiation." Drying is lethal to the unstable *Woodruffia* cysts, while the stable cysts are able to withstand drying for long periods. The question of the degree of dedifferentiation in the two types of *Woodruffia* cysts will be the subject of a future investigation.

Schewiakoff (1928) describes degrees of encystment in *Euplotes harpa*. He was able to induce various cystic forms in this organism, depending on the rate of evaporation of the medium. If the process was rapid, the organisms merely came to rest without secreting a wall. By less rapid evaporation the organisms rounded up, but only a part of them formed a thin cyst membrane. By slow evaporation, occupying 2–3 days, the organisms always formed a rigid cyst wall. All these stages could be activated. Garnjobst (1937) refers to an unidentified species of *Euplotes* which would, during evaporation of the medium, become motionless and remain alive in a stiffened condition for several days without secreting a cyst wall. The unstable and stable protective cysts of *Woodruffia* described here may, in a certain sense, be regarded as degrees of encystment. They differ in degree in the thickness of the ectocyst; and, on the basis of the evidence now at hand, they probably differ in the degree of dedifferentiation and in the amount of water present in the protoplasm. However, the idea of degrees of encystment in *Woodruffia* does not imply that the unstable type of cyst may gradually become the stable type. Close observations for over a month have not shown any such transformations.

In comparing results obtained in cystment studies on different forms, it is important to know whether the cysts can withstand drying and whether they require the presence of some organic excystment substance in the excystment medium, as well as the degree of dedifferentiation of the cysts. It is also important to know whether more than one type of cyst, with reference to these conditions, can be formed by different species. While no other detailed studies have been made on the formation of more than one type of protective cyst, other than the study of Schewiakoff referred to above, a partial review of the literature on protozoan cystment suggests that more than one type of protective cyst may be formed by other species.

In certain instances it is clear that the cysts described required some organic excystment substance for activation. Weyer (1930) attributed excystment of *Gastrostyla* to the products of bacterial action in the excystment medium. Barker and Taylor (1933) and Taylor and Strickland (1938) state that an extract of plant or animal cells is necessary for the excystment of *Colpoda*. Haagen-Smit and Thimann (1938) attributed the excysting potency of hay infusion on *Colpoda* cysts to the salts of simple organic acids present in it. Garnjobst (1937) found that the protective cysts of *Stylonethes sterkii*, which could withstand drying, required the addition of a wheat-infusion medium for excystment.

In other instances investigators have described the occurrence of excystment simply upon the addition of water. Bodine (1923) reported that excystment in *Colpoda* occurred upon the addition either of hay infusion or of tap water. Moore (1924) found that spontaneous excystment occurred in *Spathidium*. Such cysts were not viable after drying. Garnjobst (1928 and 1937) indicates that protective cysts of *Euplotes taylori* could be induced to excyst by adding either tap or distilled water. These cysts would not withstand drying. Darby (1929) caused cysts of *Stylonychia* to excyst both by adding hay infusion and by adding spring water to the preparations. In the studies of Beers (1930 and 1935) on *Didinium* it is not clear whether the cysts required an organic substrate for excystment. In this work the cysts were always transferred from either spring water or hay medium to fresh hay medium for excystment. Although it can be argued that in these cases where the addition of water caused excystment there was sufficient organic matter in the water to produce it, our experiences with the two types of protective cysts in *Woodruffia* suggest an alternative explanation—that these cysts did not require any organic excystment substance for activation.

Certain results obtained in different studies on *Colpoda* cysts suggest that an unstable type of cyst may be formed by this organism. Barker and Taylor (1933, p. 129) reported that "replacement of the encystment medium by fresh balanced medium—the same in which the organisms originally encysted—may or may not induce excystment. It occasionally does so." Thimann and Barker (1934) found that, when they diluted a preparation of *Colpoda* cysts in 0.2 cc. of balanced medium with 4.8 cc. of sterile tap water, they obtained an average excystment of 72 per cent in three such trials. They attributed this result to a dilution of the encystment medium which makes cysts less sensitive to the excystment stimulus and to the presence of traces of organic matter in the sterile tap water, which gave it a weak excysting potency. The significance of the latter suggestion is not clear in view of their other finding that the percentage of excystment depends upon the concentration and not upon the total amount of excysting material present. Haagen-Smit and Thimann (1938) experienced occasional small percentages of excystment of *Colpoda* cysts in water alone. They attributed these results to the influence of dust from the air and thereafter conducted their experiments in a constant temperature room of 24° C. with a high humidity. Such excystment in water also suggests the possibility that in these instances they were dealing with cysts comparable in behavior to the unstable type we have described here. In a recent study Taylor and Strickland (1938) found that sterile *Colpoda* isolated in a small drop of sterile balanced medium would encyst and then excyst spontaneously. Such behavior has something in common with that of the unstable cysts described here, i.e., excystment is not dependent upon some organic substance. However, the relationships, if there are any, between such *Colpoda* cysts and the unstable *Woodruffia* cysts are not clear. The very careful method used in making preparations for the standard tests made on *Colpoda* cysts by Taylor and his students in recent years would eliminate from their preparations cysts comparable to the unstable ones found in *Woodruffia*, if any such are formed. Brown and Taylor (1938, p. 477) describe their method of preparing cysts in these words: "The cyst-Cellophane preparation is cut in strips which are hung in continuously flowing balanced medium (Osterhout's, 0.012 per cent total salt) at 20° C. Under these conditions the same preparation yields uniform results for a period of many months."

It is difficult to make comparisons of results obtained in different investigations. The inferences made here are in the form of suggestions—suggestions that comparative studies should be made on a number of different cyst-forming protozoans to determine whether more than one type of protective cyst can be induced in other protozoans under reproducible conditions. If adequate generalized conclusions are to be made about protective cysts of different species of protozoa as regards their viability upon drying, their requirement of an organic excystment substance, their state of dedifferentiation, and as regards other matters of comparison, it is necessary that we know whether more than one type of protective cyst may be formed in each species, so that such conclusions will be based only on comparisons of cysts which are similar in nature. On the basis of the results presented here, it is suggested that possible criteria for distinguishing between two types of protective cysts in other organisms, purely aside from structural differences, are the ability to withstand drying and the need of plant or animal extracts in the excystment medium.

SUMMARY

Two types of protective cysts in *W. metabolica* are described. These cysts differ both morphologically and physiologically and are referred to as "stable" and "unstable" cysts. Encystment, leading to the formation of either type of cyst, is dependent upon the absence of food under the conditions of these experiments.

Excystment of the thick-walled stable cysts is dependent upon the presence of plant extracts in certain concentration in the excystment medium. The rate of excystment of stable cysts is a function of temperature, within certain limits. Excystment of such cysts is not materially affected by variations in the H-ion concentration of the excystment medium over a wide range (pH 5.5–9.0). Stable cysts will excyst in solutions with salt concentrations ranging from 0.012 to 1.2 per cent. The potency of plant extracts used for excystment is not changed by bacterial action over a period of 3 weeks. Stable cysts can withstand freezing and drying for long periods. These cysts, in a thoroughly dried condition, are killed at temperatures between 125° and 130° C., while in the wet state they are killed at temperatures between 54° and 57° C., with momentary exposures.

Crowding, the use of organisms which had fed on semistarved paramecia prior to encystment, and increasing the temperature and increasing the salt concentration of the

medium—all influenced the formation of the thin-walled unstable cysts. Excystment of these cysts could be induced by changing the balanced salt solution, by replacing the old salt solution with distilled water, or by merely lowering the temperature of the preparation from 30° to 20° C. or to room temperature. The excystment time of unstable cysts is much shorter than that of stable cysts. Unstable cysts can withstand freezing for 24 hours but are killed by thorough drying. A vacuole is always present in unstable cysts, which, together with other differences, suggests that the unstable cysts are in a less dedifferentiated state than the stable cysts.

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VOL. XIII, NO. 1, JANUARY, 1940]